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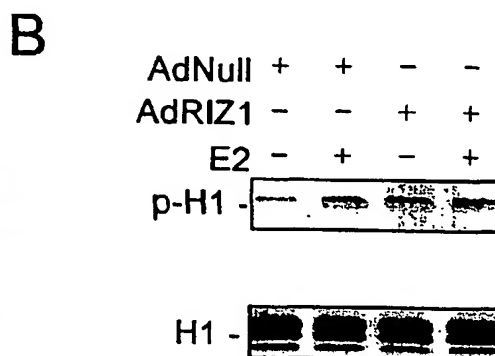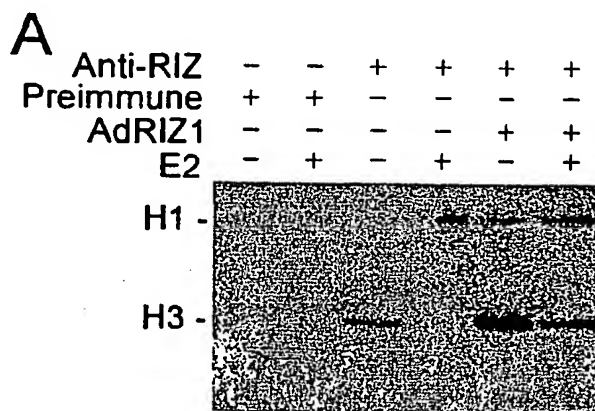
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(54) Title: SCREENING, DIAGNOSTIC AND THERAPEUTIC METHODS RELATING TO RIZ

(57) **Abstract:** The invention provides a method of screening for a compound that modulates RIZ histone methyltransferase (HMT) activity, by contacting a RIZ or RIZ fragment having HMT activity with one or more candidate compounds, and determining histone methyltransferase activity of the contacted RIZ or RIZ fragment. Also provided is a method of screening for a compound that modulates progesterone receptor (PR) activity, by providing a RIZ1 modulatory compound, and determining the ability of the RIZ1 modulatory compound to modulate PR activity. Further provided is a method of identifying an individual with an estrogen receptor positive (ER+) tumor having a reduced likelihood of responding to endocrine therapy. The method involves determining the RIZ1 status of the tumor, wherein an abnormal RIZ1 status identifies the individual as an individual with a reduced likelihood of responding to endocrine therapy.

SCREENING, DIAGNOSTIC AND THERAPEUTIC
METHODS RELATING TO RIZ

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates generally to the fields
10 of cancer biology and endocrinology and, more
specifically, to biological activities of RIZ
polypeptides.

BACKGROUND INFORMATION

Post-translational addition of methyl groups to
15 the amino-terminal tails of histone proteins is catalyzed
by a family of proteins known as histone
methyltransferases (HMTs). Histone site-specific
methylation is associated with a variety of fundamental
cellular processes, including transcriptional regulation,
20 epigenetic silencing and heterochromatin formation. Loss
of HMT function is expected to directly contribute to the
de-differentiation and genomic instability that are
characteristic of cancer.

Further implicating HMTs in cancer, it has been
shown that a deficiency in dietary methionine or folate
causes cancer. Dietary methionine and folate in turn
regulate the cellular levels of S-adenosylmethionine
5   (SAM), which is used as a methyl group donor by
methyltransferases. Additionally, several independent
lines of investigation have revealed that alterations in
the methionine metabolic pathway, which can lead to a
deficiency in SAM and/or an increase in the
10   methyltransferase inhibitor S-adenosylhomocysteine (SAH),
are associated with cancer.

Thus, there exists a need to identify proteins
with histone methyltransferase activity, determine their
substrates and regulators, and identify compounds that
15   modulate their activity. In particular, there exists a
need to identify histone methyltransferase activity
within proteins already recognized to play critical roles
in regulating cell proliferation. Compounds that
modulate the HMT activity of such proteins are expected
20   to be useful as therapeutics to regulate cell growth.

The steroid hormone progesterone is a critical
component of the female reproductive cycle and is
required to maintain pregnancy. The molecular target of
progesterone is the intracellular progesterone receptor.
25   Upon binding to progesterone, the progesterone receptor
translocates to the nucleus where it binds as a
transcription factor to DNA transcriptional elements
present in progesterone-regulated genes.

Ligands to the progesterone receptor play an
30   important role in female reproductive medicine and
cancers. For example, progesterone and its synthetic
analogs are useful in birth control formulations, in
treating endometriosis and in maintaining pregnancy.

Antagonists to progesterone are useful in treating chronic disorders such as certain forms of hormone dependent cancer of the breast, ovaries and endometrium (the lining of the uterus) and in treating uterine

5   fibroids. Progesterone antagonists are also useful in combination with other drugs for terminanting early stage pregnancies.

Compounds that modulate progesterone receptor activity are thus expected to be useful in reproductive

10 applications and for treating malignant disorders. However, suitable high-throughput assays for identifying such compounds are currently lacking, in part due to a lack of understanding of physiologically relevant interactions of the progesterone receptor with cellular

15 molecules. Thus, there exists a need for improved methods to screen for compounds that modulate progesterone receptor activity.

The steroid hormone estrogen directly and indirectly promotes the proliferation of tissues

20 expressing estrogen receptors. Endocrine therapy to reduce the proliferative effects of estrogens, including therapy with selective estrogen receptor modulators (SERMs) such as tamoxifen, is currently the preferred first-line therapy in patients with estrogen receptor

25 positive (ER+) tumors. However, a significant portion of patients with ER+ tumors do not respond to endocrine therapy. In these patients, alternative therapies are warranted in order to improve the odds of survival. Currently, there is no satisfactory method of predicting

30 which ER+ tumors will respond to endocrine therapy.

Thus, there exists a need to identify molecules that correlate with loss of estrogen responsiveness in ER+ tumors, in order to more accurately determine which

individuals are more or less likely to respond to endocrine therapy. Using such a correlation, an individualized course of treatment with an improved likelihood of success can be chosen.

5   The present invention satisfies these needs, and provides related advantages as well.

SUMMARY OF THE INVENTION

          The invention provides a method of screening for a compound that modulates RIZ histone

10   methyltransferase activity. The method involves contacting a RIZ or RIZ fragment having histone methyltransferase activity with one or more candidate compounds, and determining histone methyltransferase activity of the contacted RIZ or RIZ fragment. A

15   compound that modulates RIZ histone methyltransferase activity is identified.

          The invention also provides a method of screening for a compound that modulates progesterone receptor activity. The method involves providing a RIZ1

20   modulatory compound, and determining the ability of the RIZ1 modulatory compound to modulate progesterone receptor activity. A compound that modulates progesterone receptor activity is thereby identified.

          Also provided is a method of identifying an

25   individual with an estrogen receptor positive (ER+) tumor having a reduced likelihood of responding to endocrine therapy. The method involves determining the RIZ1 status of the tumor, wherein an abnormal RIZ1 status identifies the individual as an individual with a reduced likelihood

30   of responding to endocrine therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the effect of RIZ1 on hormone responsive promoters. The indicated amounts of RIZ1 and various nuclear hormone receptor expression vectors were
5 cotransfected with a reporter construct containing the appropriate hormone receptor response element into CV-1 cells. Cells were treated with or without the indicated hormone for 24 h and assayed for CAT activity. Data shown represent the means ± SEM of three independent
10 experiments.

Figure 1B shows that RIZ1 forms hormone-dependent protein complexes in vivo with ERα and progesterone receptor proteins. T47-D (for progesterone receptor) and MCF-7 (for ER) cells were infected with
15 adenovirus expressing RIZ1. Cell extracts treated with or without hormones were prepared and immunoprecipitated (IP) with preimmune serum or monoclonal antibodies against progesterone receptor (PR) (detecting both PR-B and PR-A) or ERα, as indicated. The immunoprecipitated
20 products were analyzed by immunoblot analysis using RIZ1, progesterone receptor or ERα monoclonal antibodies (2D7, 1A6, and sc-8005, respectively).

Figure 1C shows immunohistochemistry analysis of RIZ expression in target organs of female sex steroid
25 hormones. Tissues from 7-week old virgin wild-type mice were analyzed for expression of RIZ and ERα. The sections show RIZ and ERα immunostaining in uterus (panels a and b), breast (c and d), and vaginal epithelium (e and f). Scale bar in a, c, and e also
30 applies to b, d, and f respectively.

Figure 1D shows RT-PCR analysis of RIZ1 expression in target organs of female sex steroid

hormones.   Total RNAs isolated from the indicated tissues
of mice were used for RT-PCR analysis.

        Figure 2 shows uterine and vaginal responses to
female sex steroid hormones in RIZ1 mutant mice. A and
5   B, Uterine wet weight was measured and the ratio of
uterine weight to body weight calculated for the
indicated number of mice and presented as mean ± SEM. C.
The uterine response to a decidual response was measured.
After treatment with E2 and progesterone, and mechanical
10 stimulation of the left uterine horn, the ratio of the
weights of the stimulated (L) to the unstimulated
(control; R) horn was calculated for the indicated number
of mice and presented as mean ± SEM. D and E. Vaginal
responses. Vaginal tissues were collected and fixed for
15 H & E staining. The thickness of the entire vaginal
epithelium and the cornified layer were measured at 7
randomly selected areas at the maximum depth of the
vaginal epithelium in each mouse and data are presented
as mean ± SEM for 7 mice in each group. F and G.
20 Impaired regulation of uterine progesterone receptor
expression by E2 in RIZ1 mutant mice. Uteri were
collected and immunohistochemically analyzed for
progesterone receptor expression. Estrogen treated uteri
are shown in G (top panel: low magnification; bottom
25 panel: high magnification). Randomly selected areas were
photographed and more than 1000 cells from each
compartment of the uteri of 4 mice in each group were
counted, and data is presented as mean ± SEM (G).
Inserted scale bars apply for both genotypes.

30   Figure 3 shows mammopoiesis in RIZ1 deficient
mice. Whole mounts of the fourth mammary gland of mice
with the indicated genotypes were prepared and stained.
A and B, Seven week old virgin mice. C and D, Mice
pregnant for the first time. E and F, Higher

magnification of the ducts and alveolar structures of the
mammary glands of the pregnant mice. G and H, Mammary
glands from mice treated with hormone pellets containing
P and E2 as described in methods. I and J, Higher
5   magnification of the mammary ducts and alveolar
structures from G and H, respectively. Scale bars that
apply to both genotypes are inserted.

          Figure 4 shows modulation by RIZ of
E2-dependent transcription mediated by endogenous ER.
10   MCF-7 cells (A) and CV-1 cells (B) were cotransfected
with the reporter ERE-tk-CAT and either RIZ1 or RIZ2
expression plasmids. Cells were treated with or without
E2 and assayed for CAT activity. Data shown represent
the means ± SEM of three independent experiments.

15   Figure 5 shows methylation of histones in vitro
by the PR-domain, the PR-binding domain (PBD), and the
combined action of the two domains. Bacteria-expressed
GST fusion proteins of human RIZ1 were used for in vitro
HMT reactions using histones as substrates. Top panel
20   shows Coomassie blue staining of purified proteins and
histones. Bottom panel shows fluorography indicating
methylation of substrates. A. Methylation of H4 by
GST-RIZ200 (consisting of RIZ1 residues 1-200) but not by
GST protein alone, GST-RIZ161 (consisting of RIZ1
25   residues 1-161), and three GST-RIZ200 point mutant
proteins as indicated. B. Methylation of H4 by
GST-RIZ332 (consisting of RIZ1 residues 1-332). C.
Enhanced methylation conferred by GST-RIZ322 plus
GST-RIZPBD.

30   Figure 6 shows methylation of histones by full
length RIZ1 protein expressed in mammalian cells. A.
Nuclear extracts (500 µg) prepared from U2OS cells
infected with AdRIZ1 virus or AdNull control virus were

immunoprecipitated with preimmune serum or RIZ1 serum
anti-KG7.1S. Immunoprecipitates were then assayed for
HMT activity using free histones as substrates. The
panel shows fluorography indicating methylation of

5   histone H1, H3 and H4. B. Enhanced methylation of
lysine 9 on H3 by RIZ1 overexpression. Histones were
extracted from U2OS cells infected with AdRIZ1 or control
virus and analyzed by western blot analysis using either
antibody for methylated K9 on H3 or methylated K4 on H3.

10   C. Methylation of H3 N terminus peptide in vitro by
RIZ1. *In vitro* methylation assays using
immunoprecipitated RIZ1 as enzyme and the indicated
N-terminal 1-20 residue peptide of H3 and acetylated H3
(K9/K14 Ac: acetylated on K9 and K14 residues) as

15   substrates. The methylated peptides were resolved by 16%
Tris-Tricine SDS gel followed by fluorography. D.
Automated sequencing of the H3 N-terminal peptide
(residues 1-20) that had been methylated in vitro by full
length RIZ1 purified by immunoprecipitation. The tritium

20   incorporation of individual amino acids identified at
each successive round of microsequencing is shown.

Figure 7 shows regulation of RIZ1
methyltransferase activity by estrogen. A. Effects of
estrogen on RIZ1 in MCF7 cells. Same amounts of nuclear

25   extracts (500 µg) from E2 treated or control MCF-7 cells,
or from AdRIZ1 infected cells were immunoprecipitated
with preimmune serum or RIZ1 serum anti-KG7.1S.
Immunoprecipitates were then assayed for HMT activity
using free histones as substrates. The panel shows

30   fluorography indicating methylation of histone H1 and H3.
B. Effects of E2 and RIZ1 on H1 phosphorylation. MCF7
cells infected with control or AdRIZ1 virus were treated
with E2 or vehicle for 48 hr. Histones were extracted
after treatment and analyzed by western blot using

anti-phosph-H1 (top panel) or anti-H1 antibodies (bottom panel).

DETAILED DESCRIPTION OF THE INVENTION

The retinoblatoma-protein-interacting zinc
finger gene, or "RIZ" gene, was originally isolated in a
functional screen for proteins that bind to the Rb tumor
suppressor (Buyse et al., Proc. Natl. Acad. Sci. USA
92:4467-4471 (1995)). Two products of the RIZ gene are
produced, owing to alternative promoter usage. RIZ2 is
identical to RIZ1 except that it lacks the N-terminal 200
(rat) or 201 (human) amino acids (Liu et al., J. Biol.
Chem. 272:2984-2991 (1997)).

RIZ1 is considered to be a tumor suppressor
gene, as indicated by the following lines of evidence.
The RIZ gene maps to chromosome 1p36, one of the most
commonly deleted regions in human cancers. Gene
silencing of RIZ1, but not RIZ2, is common in many types
of human tumors - including breast cancer, liver cancer,
colon cancer, neuroblastoma, melanoma, lung cancer and
osteosarcoma (Chadwick et al., Proc. Natl. Acad. Sci. USA
97:2662-2667 (2000); He et al., Cancer Res. 58:4238-4244
(1998); Jiang et al., Int. J. Cancer 83:541-547 (1999)) -
and is associated with promoter DNA methylation (Du et
al., Cancer Res. 22:8094-8099 (2001)). RIZ1 missense
mutations (Steele-Perkins et al., Genes Dev. 15:2250-2262
(2001)) and frameshift mutations (Chadwick et al., supra
(2000); Piao et al., Cancer Res. 60:4701-47-4 (2000);
Sakurada et al., Genes, Chroms. Cancer 30:207-211 (2001))
are common in human tumors. RIZ1 expression induces
cell-cycle arrest, apoptosis induction and suppression of
xenograft tumors (Chadwick et al., supra 2000; He et al.,
supra (1998); Jiang et al., supra (1999); Jiang and
Huang, Cancer Res. 61:1796-1798 (2001)). Finally, mouse

gene knock-out models show that inactivation of RIZ1,
while retaining normal RIZ2, causes tumor susceptibility
(Steele-Perkins et al., supra (2001)). These studies
suggest an important function for the N-terminus of RIZ1
5   in its tumor suppressor activity.

     The N-terminus of RIZ1 contains a domain of
about 130 residues called the "PR-domain," between about
residues 30-160 of RIZ1. The PR-domain was initially
identified as a region that showed homology between RIZ1
10 and the transcriptional repressor PRDI-BF1. This domain
was later found to be structurally related to the
SET-domain (Huang et al., J. Biol. Chem. 273:15933-15940
(1988)), which was recently shown to be a catalytic motif
of lysine histone methyltransferases (HMTs) (Rea et al.,
15 Nature 406:593-599 (2000)). Despite the sequence
similarity between PR and SET, the PR and SET domains are
clearly distinct. First, identities among PR-domains or
among SET domains are usually about 40%, whereas
identities between PR and SET domains are typically only
20 about 20-30%. Second, SET domains are primarily found at
the carboxyl termini of proteins, whereas PR-domains are
mostly located at the amino termini. Prior to the
instant disclosure, no enzymatic activity of a PR-domain
had been described. Furthermore, histone
25 methyltransferase activity of the PR-domain of RIZ1, or
of other domains within RIZ, had not been described.

     As disclosed herein, both RIZ1 and RIZ2
proteins have histone methyltransferase activity.
30 Despite its actions as a tumor suppression, RIZ1 also
acts as an estrogen receptor (ER) and progesterone
receptor coactivator involved in regulating the normal
proliferation and differentiation of estrogen- and
progesterone-responsive tissues (see Example I). Because
35 of the dual role of RIZ as a growth suppressor and growth

promoter, compounds that modulate RIZ histone
methyltransferase activity are expected to be useful in
applications in which it desirable to positively or
negatively modulate cell growth, including treatment of
5   hyperproliferative disorders (e.g. neoplasia,
hyperplasia, inflammatory conditions and the like) and
treatment of hypoproliferative disorders (e.g. various
disorders of hematopoiesis, wound healing and the like).

The invention thus provides a method of
10   screening for a compound that modulates RIZ histone
methyltransferase activity. The method is practiced by
contacting a RIZ or RIZ fragment having histone
methyltransferase activity with one or more candidate
compounds, and assaying histone methyltransferase
15   activity of the contacted RIZ or RIZ fragment. A
compound that modulates histone methyltransferase
activity of the RIZ or RIZ fragment is thereby
identified.

20   As disclosed herein, HMT activity is present
both in the PR-domain specific to RIZ1, and also in the
C-terminal PR-binding domain (or PBD) common to RIZ1 and
RIZ2. Accordingly, either RIZ1 or RIZ2, or fragments
thereof that retain HMT activity, can be used in the
25   invention methods.

The RIZ gene from several species has been
cloned and characterized (Buyse et al., Proc. Natl. Acad.
Sci. USA 92:4467-4471 (1995); Liu et al., supra (1997)).
Accordingly, a RIZ polypeptide from any species can be
30   used in the invention screening methods. The human RIZ
gene encodes the RIZ1 amino acid sequence set forth as
SEQ ID NO:1 (GenBank Accession No. AAC50820; gi:9955379)
and the RIZ2 amino acid sequence set forth as SEQ ID
NO:2. The rat RIZ gene encodes the RIZ1 amino acid

12

sequence set forth in SEQ ID NO:3 (GenBank Accession No.
AAA74468; gi:949996) and the RIZ2 amino acid sequence set
forth as SEQ ID NO:4. The mouse RIZ gene has been
partially sequenced (U.S. Patent No. 5,811,304).

5

Human and rat RIZ1 and RIZ2 polypeptides are
highly homologous over their entire lengths (see, for
example, U.S. Patent Nos. 6,323,335; 6,069,231; and
5,811,304). Because of this high degree of identity

10 across two mammalian species, it is expected that other
naturally occurring mammalian RIZ polypeptides, such as
RIZ polypeptides from non-human primates, mouse, rabbit,
bovine, porcine, ovine, canine or feline species, as well
as naturally occurring RIZ polypeptides from other

15 vertebrates, including fish, birds, reptiles and
amphibians (e.g. *Xenopus*) will also exhibit a high degree
of identity across their lengths with human and rat RIZ.

Using knowledge of the human or rat or RIZ-
encoding nucleic acid sequences and polypeptides, those

20 skilled in the art can readily clone RIZ-encoding genes
from other species using conventional cDNA or expression
library screening methods, or using the polymerase chain
reaction (PCR). Additionally, using knowledge of the
human or rat RIZ-encoding nucleic acid sequences and

25 polypeptides, those skilled in the art can readily
determine cDNA and coding sequences from other species
from an analysis of ESTs and genomic sequences present in
available databases. From these sequences, full-length
RIZ1 or RIZ2 polypeptides, and fragments thereof with HMT

30 activity, can be prepared for use in the screening
methods disclosed herein.

A RIZ polypeptide useful in the methods of the
invention can also have one or more minor modifications
to the naturally occurring sequence, such as one or more

substitutions, additions or deletions. A modified RIZ
polypeptide will generally retain at least 70%, 75%, 80%,
90%, 95%, 98% or higher identity with a native RIZ
sequence. Such modifications can be advantageous, for

5   example, in enhancing the stability, bioavailability,
bioactivity or immunogenicity of the polypeptide, or to
facilitate its purification.

     Substitutions to a RIZ amino acid sequence can
either be conservative or non-conservative. Conservative

10   amino acid substitutions include, but are not limited to,
substitution of an apolar amino acid with another apolar
amino acid (such as replacement of leucine with an
isoleucine, valine, alanine, proline, tryptophan,
phenylalanine or methionine); substitution of a charged

15   amino acid with a similarly charged amino acid (such as
replacement of a glutamic acid with an aspartic acid, or
replacement of an arginine with a lysine or histidine);
substitution of an uncharged polar amino acid with
another uncharged polar amino acid (such as replacement

20   of a serine with a glycine, threonine, tyrosine,
cysteine, asparagine or glutamine); or substitution of a
residue with a different functional group with a residue
of similar size and shape (such as replacement of a
serine with an alanine; an arginine with a methionine; or

25   a tyrosine with a phenylalanine).

     Additions to a RIZ amino acid sequence include,
but are not limited to, the addition of "tag" sequences,
which are conveniently added at the N- or C- termini,
after the signal peptide, or within extracellular or

30   intracellular loops. Such tag sequence include, for
example, epitope tags, histidine tags, glutathione-S-
transferase (GST), fluorescent proteins (e.g. Enhanced
Green Fluorescent Protein (EGFP)) and the like. Such
additional sequences can be used, for example, to

facilitate expression, purification or characterization
of the RIZ polypeptide.

Deletions to a RIZ amino acid sequence include,
but are not limited to, deletion of residues at the N- or
5   C- termini that are not critical for function. Deleted
sequences can optionally be replaced by tag sequences or
fusion sequences, as described previously.

Modifications to a RIZ amino acid sequence can
be randomly generated, such as by random insertions,
10 deletions or substitutions of nucleotides in a nucleic
acid molecule encoding the polypeptide. Alternatively,
modifications can be directed, such as by site-directed
mutagenesis of a nucleic acid molecule encoding the
polypeptide.

15   Guidance in modifying the sequence of a RIZ
polypeptide while retaining biological activity can be
provided by the alignment of the sequence of the RIZ
orthologs from human and rat. It is well known in the
art that evolutionarily conserved amino acid residues are
20 more likely to be important for maintaining biological
activity than less well-conserved residues. Thus, it
would be expected that substituting a residue that is
highly conserved among RIZ polypeptides across species
with a non-conserved residue may be deleterious, whereas
25 making the same substitution at a residue which varies
among species would likely not have a significant effect
on biological activity. An alignment of the rat and
human RIZ1 amino acid sequences is presented in Figure 10
of U.S. Patent No. 5,811,304.

30   Computer programs well known in the art can
also provide guidance in predicting which amino acid

residues can be modified without abolishing a structural
or functional feature of a RIZ polypeptide.

As disclosed herein, RIZ histone
methyltransferase activity is present in an N-terminal,
5   PR-domain containing portion specific to RIZ1.
Accordingly, an exemplary RIZ or RIZ fragment useful in
the methods of the invention contains amino acids 1-200
of RIZ1, such as amino acids 1-332 of RIZ1. A further
exemplary RIZ or RIZ fragment useful in the methods of
10 the invention contains amino acids 1-520 of RIZ1. In
contrast, a RIZ fragment consisting of residues 1-161 of
human RIZ1 lacked HMT activity, as did a RIZ1 containing
missense mutations in the PR-domain found in tumors,
namely C106Y, I88V and A159V.

15   Additionally, as disclosed herein, RIZ histone
methyltransferase activity is also present in a C-
terminal PR-binding domain (PBD) containing portion
common to RIZ1 and RIZ2 (Huang et al., J. Biol. Chem.
273:15933-15940 (1988)). Accordingly, an exemplary RIZ
20 or RIZ fragment useful in the methods of the invention
contains amino acids 1514-1680 of RIZ1, such as amino
acids 1514 to the C-terminus of RIZ1.

The HMT activity of other RIZ polypeptides and
fragments, including polypeptides and fragment with
25 modified sequences as described above, can readily be
determined by the assays described herein in order to
select a RIZ or RIZ fragment having histone
methyltransferase activity for use in the screening
methods.

30   As used herein, the term "histone
methyltransferase activity" or "HMT activity," with
respect to a RIZ or RIZ fragment, refers to the ability

of the RIZ or RIZ fragment to catalyze the methylation of histones or histone peptides under suitable assay conditions.   In contrast, under the same conditions, a control polypeptide, such as glutathione-S-transferase

5   (GST), will not be able to catalyze the methylation of histones or histone peptides.   HMT activity can be exhibited either in an *in vitro* assay with purified or partially purified RIZ, or in a cell-based assay.   HMT activity includes HMT activity exhibited toward any

10   histone, such as histone H1, H2A, H2B, H3 or H4.

        Histones suitable for use as substrates in HMT activity assays can be obtained commercially (e.g. from Roche Molecular Biochemicals), prepared recombinantly

15   based on known nucleic acid sequences, or extracted from cells using methods known in the art.   Histone peptides suitable for use as substrates in HMT assays, including peptides with native sequences and peptides modified by acetylation on lysine residues, can be obtained

20   commercially (e.g. from Upstate Biotech) or produced synthetically.   Suitable histone peptides include, for example, H3 N-terminal peptides that include lys-9 (K9) (e.g. H3 amino acids 1-20) and H1 N-terminal peptides that include lys-25 (K25) (e.g. H1 amino acids 15-37 or

25   12-31).   The methylation site on H4 is expected to be lys-20 (K20).   Accordingly, a suitable histone H4 peptide can be an N-terminal peptide that includes lys-20.

        For HMT assays, a RIZ or RIZ fragment of any desired sequence can conveniently be produced

30   recombinantly, such as by expression of the encoding nucleic acid molecule in bacteria, yeast, insect or mammalian cells.   The expressed polypeptide can then be isolated with anti-RIZ antibodies, or purified or partially purified by standard biochemical fractionation

35   methods.   Alternatively, to facilitate isolation, the RIZ

polypeptide can be expressed as a fusion with a tag
sequence, such as glutathione-S-transferase (GST), a
6XHis tag or an epitope tag. Methods of producing and
isolating tagged and untagged recombinant proteins are
5   well known in the art (see, for example, Sambrook et al.,
Molecular Cloning: A Laboratory Manual, 3rd ed., Cold
Spring Harbor Press, Plainview, New York (2001); Ausubel
et al. (Current Protocols in Molecular Biology
(Supplement 47), John Wiley & Sons, New York (1999)).

10   As an alternative to preparing a recombinant
RIZ or RIZ fragment, an endogenous RIZ1 or RIZ2 can be
purified or partially purified from a convenient cell or
tissue source. A RIZ fragment can thus be prepared by
enzymatic or chemical cleavage of the endogenous RIZ.
15   Alternatively, a RIZ fragment can be prepared by
synthetic methods.

          HMT activity of a RIZ can be determined by
methods known in the art. For example, the RIZ and a
histone or histone peptide can be incubated with a
20   labeled methyl donor, such as
S-adenosyl-[methyl-^{14}C]-L-methionine, or
S-adenosyl-[methyl-^{3}H]-L-methionine, under suitable assay
conditions. Transfer of the radiolabel to the histone or
histone peptide can be detected, for example, by SDS-PAGE
25   electrophoresis and fluorography. Alternatively,
following the reaction the histone or histone peptides
can be separated from the methyl donor by filtration, and
the amount of radiolabel retained on the filter
quantitated by scintillation counting. Other suitable
30   labels that can be attached to methyl donors, such as
chromogenic and fluorescent labels, and methods of
detecting transfer of these labels to histones and
histone peptides, are known in the art.

Alternatively, HMT activity of a RIZ can be determined using an unlabeled methyl donor (e.g. S-adenosyl-L-methionine) and reagents that selectively recognize methylated histones or histone peptides. For

5 example, after incubation of the RIZ, methyl donor and histones or histone peptides, under suitable assay conditions, methylated histones or histone peptides can be detected by immunoblotting or by an ELISA assay with antibodies specific for methylated histone epitopes.

10 Suitable antibodies are described, for example, in Nakayama et al., <u>Science</u> 292:110-113 (2001), Noma et al., <u>Science</u> 293:1150-1155 (2001) and published U.S. Patent Application No. 20020039776, or can be prepared by methods known in the art (see Harlow and Lane,

15 <u>Antibodies: A Laboratory Manual</u>, Cold Spring Harbor Laboratory Press (1988)).

Instead of using antibodies, methylated histones can be detected using reagents that selectively bind methylated histones with high affinity. Such

20 reagents are known in the art or can be determined by screening assays known in the art. An exemplary binding reagent is heterochromatin protein HP1, which binds histone H3 when methylated at lysine 9 (H3-K9). HP1, or a binding fragment thereof, can be labeled, and the HP1

25 or fragment bound to methylated H3-K9 detected. Alternatively, the HP1 or fragment need not be labeled, and can instead be detected using an anti-HP1 antibody in an ELISA assay.

Various low-throughput and high-throughput

30 enzyme assay formats are known in the art and can be readily adapted for RIZ HMT assays. For high-throughput assays, the histone or histone peptide substrate can conveniently be immobilized on a solid support, such as a multiwell plate, slide or chip. Following the reaction,

the methylated product can be detected on the solid
support by the methods described above. Alternatively,
the HMT reaction can take place in solution, after which
the histone or histone peptide can be immobilized on a
5   solid support, and the methylated product detected. To
facilitate such assays, the solid support can be coated
with streptavidin and the histone labeled with biotin, or
the solid support can be coated with anti-histone
antibodies. The skilled person can determine suitable
10   assay formats depending on the desired throughput
capacity of the screen.

     The invention screening method involves
contacting a RIZ or RIZ fragment having histone
methyltransferase activity with one or more candidate
15   compounds and assaying histone methyltransferase activity
of the contacted RIZ or RIZ fragment. A candidate
compound useful in the methods of the invention can be a
naturally occurring macromolecule, such as a peptide,
nucleic acid, carbohydrate, lipid, or any combination
20   thereof. A candidate compound alternatively can be a
partially or completely synthetic derivative, analog or
mimetic of such a macromolecule, or a small, synthetic
molecule, such as an organic molecule prepared by
combinatorial chemistry methods. A candidate compound
25   can be detectably labeled or attached to a solid support,
if desired, as appropriate in a particular assay.

     Methods for producing large libraries of
compounds, including simple or complex organic molecules,
metal-containing compounds, carbohydrates, peptides,
30   proteins, peptidomimetics, glycoproteins, lipoproteins,
nucleic acids, antibodies, and the like, are well known
in the art and are described, for example, in Huse, U.S.
Patent No. 5,264,563; Francis et al., Curr. Opin. Chem.
Biol. 2:422-428 (1998); Tietze et al., Curr. Biol.,

2:363-371 (1998); Sofia, _Mol. Divers_. 3:75-94 (1998);
Eichler et al., _Med. Res. Rev._ 15:481-496 (1995).
Libraries containing large numbers of natural and
synthetic compounds also can be obtained from commercial
5 sources.

The number of different candidate compounds to
screen in a particular assay can be determined by those
skilled in the art, and can be 2 or more, such as 5, 10,
15, 20, 50 or 100 or more different compounds. For
10 certain applications, such as when a library of random
compounds is to be screened, and for automated
procedures, it may be desirable to screen 10^3 or more
compounds, such as 10^5 or more compounds, including 10^7 or
more compounds. If desired, a plurality of candidate
15 compounds can be assayed in a pool, and the pool
repeatedly subdivided until a single compound with the
desired activity is identified. Candidate compounds can
be assayed simultaneously, in parallel, or sequentially.

The amount of candidate compound to use in a
20 reaction can be determined by the skilled person based on
the nature of the compound, the nature of the assay, and
the concentration of the reactants. If desired, a range
of doses of candidate compound can be tested.

Generally, the candidate compound will be
25 included in an HMT reaction together with the RIZ,
histone or histone peptide substrate, and methyl donor.
Optionally, the candidate compound and the RIZ can first
be incubated together, and then the other reactants
added. If desired, other components, such as different
30 RIZ polypeptides or RIZ fragments, can be included in the
reactions, and the effect of the candidate compound on
modulating RIZ HMT activity under such conditions

determined. The skilled person can determine suitable
combinations of reactants and components.

For cell-based screening assays, a cell
expressing a RIZ can be contacted with a candidate
5   compound. Either the *in vivo* methylation of isolated
histones can be determined following contacting, or the
RIZ polypeptide can be isolated and its activity in
methylating isolated histones or histone fragments
assayed as described above. If desired, the ability of a
10 candidate compound to modulate RIZ activity under
physiologically relevant conditions can be determined in
cell-based screening assays. For example, the ability of
a candidate compound to module RIZ activity in the
presence of estrogen or prpogesterone, in response to co-
15 expressed polypeptides, or in tumor cells, can be
determined.

Determining whether a candidate compound
modulates RIZ HMT activity, either positively or
negatively, generally requires comparison to a control.
20 A control can be an identical reaction to the test
reaction, except the control is not exposed to the
candidate compound. The HMT activity of the control
reaction can be assessed either before, after, or at the
same time as the test reaction. A compound that
25 "modulates" HMT activity is a compound that increases or
decreases HMT activity, in comparison to a control, by at
least 2-fold, such as at least 5-fold, 10-fold or more.

The results described in Example I suggest that
the H3-K9 methylation activity of RIZ1 is linked with
30 growth arrest, whereas its H1-K25 methylation activity is
associated with cell proliferation. Accordingly,
compounds that differentially regulate these two
activities can be identified and used to promote or

inhibit cell growth, as appropriate for a particular application.

As disclosed herein, RIZ1, but not RIZ2, acts as a transcriptional coactivator of the nuclear hormone
5   receptors for the female sex steroids estrogen (ERα) and progesterone. Furthermore, RIZ1 is required for normal growth and development of female target organs, such as the uterus, vagina, and mammary gland, in response to estrogen and progesterone. Therefore RIZ1, and
10 particularly the PR domain of RIZ1, is a physiologically important regulator of progesterone and estrogen receptor activity. Compounds that modulate RIZ1 activity are thus likely to also be able to modulate progesterone receptor and/or ERα activity. Such compounds can be used as
15 therapeutics to prevent, ameliorate or treat conditions that are benefitted by modulated female sex steroid receptor activity, including reproductive conditions and cancer.

In one embodiment, the invention provides a
20 method of screening for a compound that modulates progesterone receptor activity. The method is practiced by providing a RIZ1 modulatory compound, and determining the ability of the compound to also modulate progesterone receptor activity.

25   As used herein, the term "RIZ1 modulatory compound" refers to a compound that affects a RIZ1 biological activity, such as a binding interaction with a cellular molecule or an enzymatic activity (e.g. histone methyltransferase activity). A RIZ1 modulatory compound
30 can thus be, for example, a compound that selectively binds RIZ1, a compound that modulates (by either increasing or decreasing) an interaction between RIZ1 and a binding partner, a compound that modulates a functional

activity of RIZ1, or a compound with several of these
effects.

Compounds that selectively bind RIZ1 are known
in the art or can be identified by available screening
5 methods. For example, compounds that selectively bind
RIZ1 include RIZ1-specific antibodies (described, for
example, in U.S. Patent No. 5,811,304) and RIZ1 binding
partners. As used herein, the term "RIZ1 binding
partner" is intended to refer to a cellular molecule that
10 normally binds with high affinity to a RIZ1, such as
cellular proteins, nucleic acid molecules, enzymatic
substrates and cofactors, the RIZ1-binding domains from
these molecules, and RIZ1-binding variants and analogs of
these molecules.

15   A "variant" of a RIZ1-binding partner has
substantially the same amino acid or nucleotide sequence
as the native molecule (e.g. at least 70%, 75%, 80%, 90%,
95%, 98% or higher identity), and retains substantially
the same binding activity as the native molecule. In the
20 case of a polypeptide, the polypeptide can have one or
more conservative or non-conservative amino acid
substitutions, additions or deletions compared to the
naturally occurring sequence. Methods of preparing and
assaying the binding activity of variants of RIZ1-binding
25 partners are well known in the art.

An "analog" of a RIZ1-binding partner has
substantially the same core chemical structure as the
native molecule, and retain substantially the same
binding activity as the native molecule. Methods of
30 preparing and assaying the binding activity of analogs of
RIZ1-binding partners are well known in the art.

An exemplary cellular protein that binds RIZ1 is the retinoblastoma protein (Rb). U.S. Patent No. 5,811,304 describes the specific binding of RIZ1 to full-length Rb and to a C-terminal 56 kDa fragment of Rb that contains the Rb binding pocket (see also Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995)). Other cellular proteins that selectively bind RIZ1 include the estrogen receptor (ERα) (see Abbondanza et al., Proc. Natl. Acad. Sci. USA 97:3130-3135 (2000) and Example I) and progesterone receptor (PR) (see Example I). Additionally, GATA-3 (Shapiro et al., Gene 163:329-330 (1995)), p53, SRC1 and p300 are exemplary cellular proteins that selectively bind RIZ1.

Nucleic acid molecules that selectively bind RIZ1 are described, for example, in U.S. Patent No. 5,811,304. Exemplary nucleic acid molecules to which RIZ1 selectively binds, through its zinc finger DNA binding motifs, contain GC-rich or Sp-1-binding elements.

Exemplary enzymatic substrates and cofactors that selectively bind RIZ1 include GTP (see U.S. Patent No. 5,811,304), zinc ions (which associate with the zinc finger motifs) and S-adenosyl-methionine (SAM).

SAM acts as a substrate and methyl group donor for RIZ1 methyltransferase activity. Analogs of SAM include S-adenosyl-L or D-homocysteine (SAH), and adenosyl-L-ethionine. Other binding compounds can include, for example, A-adenosyl-gamma-thio-alpha-ketobutyrate, S-adenosyl-L-homocysteine sulfoxide, methythioadenosine (MTA), L-homocysteine, L-homoserine, Adenosine, Adenine, ATP, CAMP and methionine.

Therefore, any of the above compounds, or variants, analogs, agonists and antagonists of these

compounds, can be assayed for their ability to modulate
progesterone receptor activity by the methods of the
invention.

Compounds that modulate the interaction between
5   RIZ1 and a binding partner are also known in the art or
can be identified by available screening methods. For
example, U.S. Patent No. 5,811,304 describes a 17 amino
acid Rb binding peptide (101-118: T-pep) from the SV40
large T antigen as a compound that interferes with the
10 interaction between RIZ1 and Rb. Other molecules that
bind to a RIZ1 binding partner, or include a binding
domain from a RIZ1 binding partner, are expected to be
compounds that modulate the interaction between RIZ1 and
a binding partner. Other compounds that can modulate the
15 interaction between RIZ1 and a binding partner can
contain a RIZ1 binding domain, and thus compete with
full-length RIZ1 for the binding partner. RIZ binding
domains include, for example, the LXXLL steroid receptor
binding motif described herein, as well as the
20 E1A-related region; the leucine-zipper; conserved regions
1 and 2; common epitope 1; zinc fingers; the GTPase
domain; the SH3 domain; the SH3-binding domain; and the
PMT (PR) domain, each of which is described in U.S.
Patent No. 5,811,304.

25   Other compounds that selectively bind RIZ1, or
that modulate the interaction between RIZ1 and a binding
partner, can be identified by either manual or high-
throughput screening assays, starting from a library of
known or unknown compounds, as described above.

30   A binding assay can use a detectably labeled
candidate compound and an unlabeled RIZ1 (and optionally
an unlabeled binding partner). Alternatively, a binding
assay can use an unlabeled candidate compound or binding

partner and a labeled RIZ1. Other appropriate combinations of labeled and unlabeled molecules can be determined by the skilled person depending on the assay format.

5   A variety of competitive and non-competitive binding assay formats for determining binding between molecules are known in the art. These assays include both solution-based methods and solid phase methods (e.g. molecules bound to plates, chips, affinity columns and

10   the like). Binding assays are amenable to either manual or high-throughput automated screening of compounds. Two exemplary binding assays are set forth in Example IIA and IIB.

          Detectable labels can include, for example, a

15   radioisotope, fluorochrome, ferromagnetic substance, or luminescent substance. Exemplary radiolabels useful for labeling compounds include ^{125}I, ^{14}C and ^{3}H. Methods of detectably labeling organic molecules, either by incorporating labeled amino acids into the compound

20   during synthesis, or by derivatizing the compound after synthesis, are known in the art.

          RIZ1 functional activities are described herein or are known in the art. Exemplary activities include, for example, transcriptional activation (see, for

25   example, U.S. Patent No. 5,811,304), transcriptional repression (see, for example, Xie et al., J. Biol. Chem. 272:26360-26366 (1997)), histone methyltransferase activity (see Example I) and hormone receptor coactivation (see Examples I and II).

30   Suitable assays for identifying compounds that modulate RIZ1 transcriptional activation, repression and coactivation function can be determined by the skilled

person. Such assays are generally based on co-expression
of RIZ1 and an appropriate promoter-linked reporter gene
in a cell, under conditions where a certain amount of
transcription occurs, contacting the cell with the
5   candidate compound, and determining whether there is a
change (i.e. either an increase or decrease) in
transcriptional activity. Transcription based assays are
well known in the art, and readily amenable to high-
throughput screening assays. Methyltransferase activity
10   assays have been described above.

A compound that is identified as a RIZ1
modulatory compound by any of the above methods is then
tested, in either an *in vitro* or *in vivo* assay, to
determine whether it also modulates progesterone receptor
15   activity. A compound that "modulates" progesterone
receptor activity is intended to refer to a compound that
either increases or decreases a PR biological activity.

The progesterone receptor is normally present
in an inactive form in the cytoplasm; where it interacts
20   with molecular chaperones, immunophillins, and heat shock
proteins. The active PR binds progesterone and
translocates to the nucleus where it binds as a
transcription factor to canonical DNA transcriptional
elements present in progesterone-regulated genes. As
25   described herein, RIZ1 coactivates the transcriptional
activity of PR. A compound that modulates progesterone
receptor (PR) activity can thus be, for example, a
compound that alters the interaction between PR and the
molecules that normally hold it in an inactive form;
30   alters the interaction between PR and progesterone;
alters the interaction between RIZ1 and PR; or directly
increases or decreases the transcriptional activity or
specificity of PR toward PR-regulated genes.

Competitive and non-competitive binding assays
have been described above with respect to RIZ1, and can
be applied to identifying compounds that modulate PR
interactions with progesterone, regulatory molecules or
5   RIZ1. Assays that measure PR transcriptional activity
can also be used to identify compounds that modulate PR
activity. Conveniently, the progesterone response
element (PRE) can be operatively linked to a reporter
gene (e.g. luciferase, green fluorescent protein, beta-
10   galactosidase and the like) and expressed in a cell, and
an increase or decrease in expression of the reporter
gene in response to a compound determined. Such an assay
is described in Example I and IIC. Alternatively, a gene
that is naturally induced by PR can be used in such
15   assays, such as casein, and the mRNA or protein product
detected.

Transcription-based assays, reporter genes, and
methods for detecting gene expression, and methods of
adapting these assays for high-throughput screening are
20   well known in the art and described, for example, in
Ausubel et al., Current Protocols in Molecular Biology,
John Wiley and Sons, Baltimore, MD (2001).

Other assays for compounds that modulate
progesterone receptor activity include in vivo assays
25   that report PR function, such as the assays for uterine,
vaginal and mammary development described in Example I
and IID. A change in development of these organs in
response to treatment of the animal with a particular
compound is consistent with the compound having an effect
30   on PR activity.

Once identified, compounds that modulate
progesterone receptor activity can be formulated as
pharmaceutical compositions and used to prevent or treat

reproductive and endocrinological disorders and cancers.
Such disorders can include, for example: infertility,
tubal disease, ovulatory defects, endometriosis,
disruptions of the estrous cycle, disruptions of the

5 menstrual cycle, polycystic ovary syndrome, ovarian
hyperstimulation syndrome, endometrial and ovarian
tumors, autoimmune disorders, ectopic pregnancy,
teratogenesis, cancer of the breast, fibrocystic breast
disease, galactorrhea, disruptions of spermatogenesis,

10 abnormal sperm physiology, cancer of the testis, cancer
of the prostate, benign prostatic hyperplasia,
prostatitis, carcinoma of the male breast and
gynecomastia. Other conditions in which increasing or
decreasing progesterone receptor activity would be

15 beneficial are known in the art or can be determined by
the skilled person. The invention thus provides for the
administration of a pharmaceutical composition containing
a compound that modulates progesterone receptor activity,
to prevent or ameliorate any of the disorders and

20 conditions described above.

     The pharmaceutical compositions of the
invention, which include compounds that modulate RIZ1 HMT
activity, and compounds that modulate progesterone
receptor activity, may be administered alone or in

25 combination with at least one other agent, such as
stabilizing compound, which may be administered in any
sterile, biocompatible pharmaceutical carrier, including,
but not limited to, saline, buffered saline, dextrose,
and water. The compositions may be administered to a

30 patient alone, or in combination with other agents, drugs
or hormones.

     The pharmaceutical compositions utilized in
this invention may be administered by any number of

routes including, but not limited to, oral, intravenous,
intramuscular, intra-arterial, intramedullary,
intrathecal, intraventricular, transdermal, subcutaneous,
intraperitoneal, intranasal, enteral, topical,
5 sublingual, or rectal means.

In addition to the active ingredients, these
pharmaceutical compositions may contain suitable
pharmaceutically-acceptable carriers comprising
excipients and auxiliaries which facilitate processing of
10 the active compounds into preparations which can be used
pharmaceutically. Further details on techniques for
formulation and administration may be found in the latest
edition of Remington's Pharmaceutical Sciences (Maack
Publishing Co., Easton, Pa.).

15 Pharmaceutical compositions for oral
administration can be formulated using pharmaceutically
acceptable carriers well known in the art in dosages
suitable for oral administration. Such carriers enable
the pharmaceutical compositions to be formulated as
20 tablets, pills, dragees, capsules, liquids, gels, syrups,
slurries, suspensions, and the like, for ingestion by the
patient.

Pharmaceutical preparations for oral use can be
obtained through combination of active compounds with
25 solid excipient, optionally grinding a resulting mixture,
and processing the mixture of granules, after adding
suitable auxiliaries, if desired, to obtain tablets or
dragee cores. Suitable excipients are carbohydrate or
protein fillers, such as sugars, including lactose,
30 sucrose, mannitol, or sorbitol; starch from corn, wheat,
rice, potato, or other plants; cellulose, such as methyl
cellulose, hydroxypropylmethyl-cellulose, or sodium
carboxymethylcellulose; gums including arabic and

tragacanth; and proteins such as gelatin and collagen.
If desired, disintegrating or solubilizing agents may be
added, such as the cross-linked polyvinyl pyrrolidone,
agar, alginic acid, or a salt thereof, such as sodium
5   alginate.

Pills, capsules, tablets and the like may
further contain suitable coatings, such as concentrated
sugar solutions, which may also contain gum arabic, talc,
polyvinylpyrrolidone, carbopol gel, polyethylene glycol,
10 and/or titanium dioxide, lacquer solutions, and suitable
organic solvents or solvent mixtures. Dyestuffs or
pigments may be added to the pills or coatings for
product identification or to characterize the quantity of
active compound, i.e., dosage.

15   Pharmaceutical preparations which can be used
orally include push-fit capsules made of gelatin, as well
as soft, sealed capsules made of gelatin and a coating,
such as glycerol or sorbitol. Push-fit capsules can
contain active ingredients mixed with a filler or
20 binders, such as lactose or starches, lubricants, such as
talc or magnesium stearate, and, optionally, stabilizers.
In soft capsules, the active compounds may be dissolved
or suspended in suitable liquids, such as fatty oils,
liquid, or liquid polyethylene glycol with or without
25 stabilizers.

Pharmaceutical formulations suitable for
parenteral administration may be formulated in aqueous
solutions, preferably in physiologically compatible
buffers such as Hanks' solution, Ringer's solution, or
30 physiologically buffered saline. Aqueous injection
suspensions may contain substances which increase the
viscosity of the suspension, such as sodium carboxymethyl
cellulose, sorbitol, or dextran. Additionally,

suspensions of the active compounds may be prepared as
appropriate oily injection suspensions. Suitable
lipophilic solvents or vehicles include fatty oils such
as sesame oil, or synthetic fatty acid esters, such as
ethyl oleate or triglycerides, or liposomes. Non-lipid
polycationic amino polymers may also be used for
delivery. Optionally, the suspension may also contain
suitable stabilizers or agents which increase the
solubility of the compounds to allow for the preparation
of highly concentrated solutions.

        For topical or nasal administration, penetrants
appropriate to the particular barrier to be permeated are
used in the formulation. Such penetrants are generally
known in the art.

        The pharmaceutical compositions of the present
invention may be manufactured in a manner that is known
in the art, e.g., by means of conventional mixing,
dissolving, granulating, dragee-making, levigating,
emulsifying, encapsulating, entrapping, or lyophilizing
processes.

        The pharmaceutical composition may be provided
as a salt and can be formed with many acids, including
but not limited to, hydrochloric, sulfuric, acetic,
lactic, tartaric, malic, succinic, etc. Salts tend to be
more soluble in aqueous or other protonic solvents than
are the corresponding free base forms. In other cases,
the preferred preparation may be a lyophilized powder
which may contain any or all of the following: 1-50 mM
histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH
range of 4.5 to 5.5, that is combined with buffer prior
to use.

After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling can include amount, frequency, and method

5   of administration.

Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an

10 effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, or in animal models, usually mice, rats, rabbits,

15 dogs, or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

20   A therapeutically effective dose refers to that amount of active ingredient which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the

25 dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions which exhibit

30 large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably

34

within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of
5   administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active
10 moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s),
15 reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

20   Normal dosage amounts may vary from 0.1 µg to 100 mg, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in
25 the art.

As dislosed herein, RIZ1 is an estrogen receptor coactivator. RIZ1 silencing is significantly more common in ER-negative breast cancers compared with ER-positive breast cancers, and appears to be implicated
30 in the acquisition of estrogen resistance, since nearly half of ER+ cancer tissues examined were determined to be RIZ1 expression-negative.

The action of estrogen is mediated via
interaction with a specific receptor (ER) that initiates
a series of downstream events, leading to the modulation
of hormone-responsive genes and cell proliferation.
5   Endocrine therapy, including both surgical and medical
therapies to reduce estrogen action, is the preferred
first-line therapy in patients with ER+ cancers.

However, a large percentage of patients with
ER-positive tumors do not respond to endocrine therapy.
10 The determination that RIZ1 inactivation is highly
associated with decreased estrogen responsiveness in
breast cancer cells allows RIZ1 status to be used to
predict whether an individual with an ER+ tumor is likely
to respond to endocrine therapy.

15   Based on information regarding RIZ1 status, a
clinician can determine a suitable treatment for the
particular individual that is more likely to be effective
in prolonging disease-free survival and/or reducing
mortality, while avoiding exposing the individual to
20 unnecessary treatments with potential side effects. For
an individual determined by the invention method to have
a normal likelihood of responding to endocrine therapy,
endocrine therapy is appropriate. However, for an
individual determined by the invention method to have a
25 reduced likelihood of responding to endocrine therapy, an
alternative treatment can be chosen from among the
available treatment options. Alternatively, for such an
individual, endocrine therapy can be combined with an
additional treatment.

30   The nature of an alternative or additional
treatment for such an individual can be chosen by the
clinician depending on the type and stage of tumor,
extent of metastasis, overall health of the individual

and other concurrent treatments. For example, in an individual determined to have a reduced likelihood of responding to endocrine therapy, chemotherapy, surgery or radiation can be combined with, or used as an alternative

5   to, endocrine therapy.

Accordingly, the invention provides a method of identifying an individual with an estrogen receptor positive (ER+) tumor having a reduced likelihood of responding to endocrine therapy. The method is practiced

10 by determining the RIZ1 status of the tumor, wherein an abnormal RIZ1 status identifies the individual as an individual with a reduced likelihood of responding to endocrine therapy. A normal RIZ1 status identifies the individual as an individual with a normal likelihood of

15 responding to endocrine therapy.

As used herein, the term "endocrine therapy" refers to a therapy that reduces the proliferative potential of estrogen. Endocrine therapy can be either surgical or medical, and affect any aspect of estrogen

20 action, including estrogen production and estrogen receptor signaling.

Methods of ablating estrogen production include surgical removal or irradiation of the ovaries, which are the primary source of estrogen in premenopausal women.

25 Ablation of estrogen production can also be achieved pharmacologically by using luteinizing hormone-releasing hormone or gonadotropin-releasing hormone agonists.

Selective estrogen receptor modulators (SERMs) refer to compounds that blunt or block the effects of an

30 estrogen agonist, such as 17β-estradiol, when administered concomitantly in a test system. SERMs used in cancer therapy, although commonly known as "anti-

estrogens," generally exhibit a combination of anti-
estrogenic and estrogenic effects. The antagonist effect
of tamoxifen, the most widely used SERM, is a result of
blocking the transmission of E2-ER-initiated signals for
5   cell proliferation. Other SERMs include
4-hydroxytamoxifen and related triphenylene
antiestrogens; clomiphene; and non-uterotrophic
antiesestrogens, such as raloxifene, droloxifene,
idoxifine, nafoxidine, toremifene, TAT-59,
10   levomeloxifene, LY-353381, CP-336156, MDL-103323, EM-800,
ERA-923, ICI-182,780 and the like. Other SERMs are known
in the art or can be determined.

Aromatase inhibitors are also used in endocrine
therapy for ER+ cancers in postmenopausal women.
15   Aromatase catalyzes the final step in the synthesis of
the estrogens estrone and estradiol from androgens and,
therefore, inhibitors of aromatase reduce estrogen
production. Exemplary aromatase inhibitors include
aminoglutethimide, megestrol acetate, anastrozole,
20   letrozole and exemestane.

As used herein, the term "estrogen receptor
positive (ER+) tumor" refers to a tumor that expresses
estrogen receptors above an art-recognized threshold
level. A number of assays are routinely used in the art
25   to categorize a tumor as ER+ or ER-. Such assays
include, for example, ligand binding assays (LBA) and
immunohistochemistry (IHC). Conveniently,
immunohistochemistry can allow for simultaneous
determination of ER status and RIZ1 status of a tumor, as
30   described herein. Generally, an ER level of greater than
3fmol/mg cytosolic protein by an LBA is considered ER+,
whereas greater than 1% positive cells by an IHC assay is
considered ER+ (Harvey et al., J. Clin. Oncol. 17:1474-
1481 (1999); Elledge et al., Int. J. Cancer (Pred.

Oncol.) 89:111-117 (2000)). A cutoff of 10% positive
cells was considered ER+ in the immunohistochemical
assays described in Example I. However, the results
would not have changed significantly using a 1% cutoff.

5   As used herein, the term "tumor" refers to a
malignant neoplasm. A tumor can be either a primary
lesion or a metastatic lesion. Tumors that have been
shown to express estrogen receptors include breast
carcinoma, endometrial carcinoma, prostate carcinoma,
10   ovarian carcinoma, renal carcinoma, melanoma, colorectal
tumors, desmoid tumors, pancreatic carcinoma, and
pituitary tumors. The methods of the invention are
applicable to ER+ tumors of any of these types, as well
as other tumors determined to be ER+. The methods of the
15   invention are applicable to ER+ cancers at any clinical
stage, including localized cancers, cancers with regional
spread and cancers with distant spread.

          Additionally, the method can be practiced with
respect to individuals whose tissues upon biopsy are
20   determined not to be malignant, including tissues that
are determined to be normal or precancerous. An abnormal
RIZ1 status in such ER+ tissues is predictive of an
increased likelihood of the individual developing a tumor
in that tissue. In particular, in women with low amounts
25   of circulating estrogen, including post-menopausal women
and women whose ovaries have been removed, an abnormal
RIZ1 status in ER+ tissues is predictive of an increased
likelihood of the individual developing a tumor in that
tissue. For such individuals, prophylactic treatment,
30   other than endocrine therapy, can be warranted. Suitable
prophylactic treatments, including lifestyle changes
(e.g. diet and exercise) and drug treatments, can be
determined by those skilled in the art.

As used herein, the term "status" with respect to RIZ1 refers to a measurable property that is correlated with RIZ1 polypeptide function in the tumor. The measurable property that is determined can be a
5   property of the RIZ1 polypeptide or of the encoding RIZ1 gene or mRNA.

An exemplary measurable property correlated with RIZ1 polypeptide function in the tumor in the tumor is the amount of the RIZ1 gene, mRNA or polypeptide in
10   the tumor. An "abnormal RIZ1 status" in this case can thus be a decreased amount, in the tumor sample, of the RIZ1 gene, mRNA or polypeptide. Generally, a decreased amount of the RIZ1 gene, mRNA or polypeptide in the tumor refers to at least an 80% reduction, such as a 90%
15   reduction, in the amount of the RIZ1 gene, mRNA or polypeptide in the tumor sample, relative to the amount in a control sample. The skilled person can determine an appropriate control for a particular assay, such as nearby normal tissue from the same individual, or a tumor
20   sample previously identified as having a normal amount of the RIZ1 gene, mRNA or polypeptide.

A further exemplary measurable property correlated with RIZ1 polypeptide function in the tumor is the structural integrity of the RIZ1 gene. An "abnormal
25   RIZ1 status" in this case can thus be a mutation in the normal nucleotide sequence of the RIZ1 gene in the tumor sample. Such a mutation can be determined directly by sequencing all or selected portions of the RIZ1 gene or mRNA. A mutation in the RIZ1 gene sequence can also be
30   inferred indirectly, such as by detecting an alteration in the normal size or abundance of the RIZ1 mRNA, an alteration in the normal ratio of RIZ2/RIZ1, or by detecting an alteration in the normal size, stability or localization of the RIZ1 polypeptide product. Mutations

that can affect the ability of the RIZ1 polypeptide to be
expressed or function normally include, for example,
genomic deletions, frameshift mutations (Chadwick et al.,
Proc. Natl. Acad. Sci. USA 97:2662-2667 (2000); Piao et

5 al., _Cancer Res_. 60:4701-4704 (2000); Sakurada et al.,
Genes, Chroms. Cancer 30:207-211 (2001)), missense
mutations, nonsense mutations, and mutations in
regulatory regions (U.S. Patent No. 5,811,304).

Methods for determining the amount or

10 structural integrity of a particular gene, mRNA or
polypeptide in a sample are well known in the art and
described, for example, in Sambrook et al., _Molecular
Cloning: A Laboratory Manual_, 3rd ed., Cold Spring Harbor
Press, Plainview, New York (2001); Ausubel et al.

15 (_Current Protocols in Molecular Biology_ (Supplement 47),
John Wiley & Sons, New York (1999)). The particular
method for a given application can be chosen by the
skilled person. Exemplary methods include, for example,
DNA sequencing, SSCP, Southern blotting, PCR, Northern

20 blotting, RT-PCR, RNAse protection, _in situ_
hybridization, immunohistochemistry, immunoblotting and
immunoprecipitation. Reagents suitable for detecting
RIZ1 nucleic acid molecules in these methods, such as
hybridization probes and PCR primers, are known in the

25 art and described, for example, in He et al., _Cancer Res_.
4238-4244 (1998) and in the Example herein. Reagents
suitable for detecting RIZ1 polypeptide in these methods,
such as the KG7.1S and 2D7 antibodies, are also known in
the art and described, for example, in Buyse et al. _Proc._

30 _Natl. Acad. Sci. USA_ 92:4467-4471 (1995).

Another exemplary measurable property
correlated with RIZ1 polypeptide function in the tumor is
the extent of methylation of the RIZ1 gene promoter. As
described in Du et al., _Cancer Res_. 61:8094-8099 (2001),

partial or complete methylation of the 35 CpGs in the
RIZ1 promoter is strongly correlated with reduced RIZ1
mRNA expression, which in turn results in reduced RIZ1
polypeptide abundance. An "abnormal RIZ1 status" in this
5   case can thus be partial or complete RIZ1 promoter CpG
methylation.

       RIZ1 promoter methylation can be determined,
for example, by the methods described in Du et al., _supra_
(2001). Briefly, one method involves treating genomic
10 DNA from a sample with bisulfite, amplifying the RIZ1
promoter DNA using polymerase chain reaction (PCR),
followed by cloning and sequencing the amplified product.
Bisulfite treatment converts unmethylated cytosines to
uridine, whereas methylated cytosines are resistant to
15 conversion. An exemplary primer set suitable for RIZ1
promoter amplification by this method is 5'-
GGTTGGGTGGTGGTTATTGGG-3' (SEQ ID NO:5) and 5'-
CAAAAACCGCCCTGCGCCACTCCTTACC-3' (SEQ ID NO:6). An
alternative method involves treating genomic DNA from a
20 sample with bisulfite and amplifying the DNA using
methylation-specific polymerase chain reaction (MSP) with
RIZ1 specific primers. An exemplary primer set for
selectively amplifying methylated RIZ1 promoter DNA is
5'-GTGGTGGTTATTGGGCGACGGC-3' (SEQ ID NO:7) and 5'-
25 GCTATTTCGCCGACCCCGACG-3' (SEQ ID NO:8), whereas an
exemplary primer set for selectively amplifying
unmethylated RIZ1 promoter DNA is 5'-
TGGTGGTTATTGGGTGATGGT-3' (SEQ ID NO:9) and 5'-
ACTATTTCACCAACCCCAAGA-3' (SEQ ID NO:10).

30   Another exemplary measurable property
correlated with RIZ1 polypeptide function in the tumor is
histone methyltransferase activity of the RIZ1
polypeptide. An "abnormal RIZ1 status" in this case can
thus be altered H1-K25 methylation activity, altered

H3-K9 methylation activity, or an alteration in the
normal RIZ activity toward another identified histone
methylation site. Such an alteration can be either
increased or decreased HMT activity, relative to a normal
5   control. Histone methyltransferase assays have been
described above, and can be adapted by the skilled person
for determining histone methyltransferase activity of a
RIZ1 polypeptide in a tumor sample.

        The skilled person can determine other
10 measurable properties correlated with RIZ1 polypeptide
function in the tumor, which can be used in the invention
method to determine RIZ1 status. If by any of these
criteria it is determined that the RIZ1 polypeptide would
be unlikely to function normally in the tumor, whether
15 because of altered amount, structure or activity, the
tumor is designated as having an "abnormal RIZ1 status,"
and the individual identified as having a "reduced
likelihood of responding to endocrine therapy."
Accordingly, alternatives to endocrine therapy, or the
20 use of endocrine therapy in combination with other
therapies, are warranted in such an individual to improve
the chance of disease-free and overall survival.

        The following examples are intended to
illustrate but not limit the present invention.

25   EXAMPLE I

        This example shows that the tumor suppressor
gene RIZ1(PRDM2) is a member of the histone
methyltransferase superfamily and a coactivator of female
sex hormone receptors. This example also shows that RIZ1
30 mediates female sex hormone action *in vivo*, and that loss
of its function is involved in the transition to hormone

resistance and hormone independent growth of breast
cancer.

Materials and Methods

Plasmids and transient transfections

5   The human RIZ1 and RIZ2 expression vectors and
RIZ1 mutant vectors used are described in Steele-Perkins
et al., Genes Dev. 15:2250-2262 (2001). The mammalian
RIZ1 expression vector p3RIZ1RH4.1 was constructed by
cloning the full-length wild type human RIZ1 cDNA into
10   the pcDNA3 vector (Invitrogen). The vector
p3RIZ1RH4.1-L965A containing a missense mutation (leucine
to alanine) was generated by the Quick-Change mutagenesis
kit (Stratagene), and verified by DNA sequencing.
Expression vectors of RIZ proteins and expression vectors
15   containing full length mammalian ERα, PR, RARα, RXRα,
VDR, AR, TR and GR were cotransfected into CV-1 cells
with an appropriate reporter construct containing a
synthetic hormone response element linked to the tk-CAT
reporter. The reporter TREpal-tk-CAT containing a
20   synthetic response element for RAR, RXR and TR (Zhang et
al., Nature 355:441-446 (1992)) was used to evaluate
effects of RARα, RXRα, and TR, the GRE-tk-CAT (Zhang et
al., J. Biol. Chem. 266:8248-8254 (1991)) for GR and AR,
the ERE-tk-CAT (Lee et al., Mol. Cell. Biol. 15:4194-4207
25   (1995)) for ERα, the PRE-tk-cat for PR (McKay and
Cidlowski, Mol. Endocrinol. 12:45-56 (1998)), and the
VDRE-tk-CAT (Agadir et al., Carcinogenesis 20:577-582
(1999)) for VDR.

          A calcium phosphate precipitation procedure was
30   used for transient transfection as described previously
(Lee et al.,Mol. Cell. Biol. 15:4194-4207 (1995)).
Briefly, 0.5-1.0x10^5 cells/well were seeded in 24-well

plates, and 50-400 ng of RIZ plasmids, 100 ng of
expression vectors for NHRs, 100 ng of reporter plasmid,
and 100 ng of a ß-gal expression vector were mixed with
carrier DNA to 1 µg of total DNA/well. Transfections of

5   MCF-7 cells (2x10⁵ cells/well in 6-well plates) used the
Effectene Transfection Reagent (Qiagen), according to the
manufacture's instructions. Cells were treated with or
without the indicated hormone (50 nM for estradiol (E2),
and 100 nM for R5020 (synthetic P agonist), all-trans

10 retinoic acid (all-trans-RA), 9-cis retinoic acid
(9-cis-RA), 1,25 dihydroxyvitamin D3 (VD3),
dihydrotestosterone (DHT), triiodothyronine (T3), and
dexamethasone (DEX); all from Sigma except R5020 which
was from NEN) for 24 h. and CAT activity was measured as

15 described (Lee et al., Mol. Cell. Biol. 15:4194-4207
(1995)). CAT values were normalized for transfection
efficiency by the corresponding ß-gal activity.

Plasmids used for GST protein production were
constructed by PCR cloning as follows. To express human

20 RIZ1 protein 1-200 residue region, full length human RIZ1
cDNA plasmid was used as template for PCR (Pfu
polymerase, Stratagene) by primers hRP109.2 (AAA CCA TGG
ATC AGA ACA CTA CTG AG (SEQ ID NO:11)) and RP274 (CCG TAA
GCT TCA TGC AGA GGT GAA ATC TGG C (SEQ ID NO:12)). The

25 PCR fragment was cloned into the NcoI and HindIII sites
in the vector pKG-PBR (Huang et al., J. Biol. Chem.
273:15933-15940 (1998b)) to generate pKG-HN1. BL-21
cells were transformed by pKG-HN1 to produce the
GST-RIZ200 protein. To express RIZ1 protein 1-161

30 residue region, a stop codon was introduced at residue
162 in the plasmid pKG-HN1 by using the Quick-Change
mutagenesis kit (Stratagene). The primers used were
RP296P1 (CGA GCC AGC GCC CGG AGC TAA GCT TAA GCG GAG CTC
CCC (SEQ ID NO:13)) and RP296P2 that has the compliment

35 sequence of RP296P1. To generate missense mutant

GST-RIZ200 proteins, mutagenesis by Quick-Change was
performed on the pKG-HN1 plasmid template using primers
as described previously (Steele-Perkins et al., Genes
Dev. 15:2250-2262 (2001)). To generate longer peptide
5 fragments of RIZ1 in bacteria, the N-terminal 520-residue
fragment was first cloned into the Nco1 and HindIII sites
of pKG-PBR vector by PCR to generate the plasmid pKGH205.
However, this plasmid did not give high yields of the
expected protein product. Next, a stop codon was
10 introduced into this plasmid by Quickchange mutagenesis
to generate pKGRIZ332, which then produced high yields of
the RIZ1 N-terminus 332-residue peptide. The PCR primers
used for mutagenesis are: RP307: GAT TTA TTA GAG GAA tgA
AAA ACA ACT TCA GAA G (SEQ ID NO:14), and RP308: CTT CTG
15 AAG TTG TTT TTc aTT CCT CTA ATA AAT C (SEQ ID NO:15).

Immunoprecipitation and Immunoblot

       MCF-7 and T47-D breast cancer cells were grown
in 15 cm dishes in DMEM with 5 % FCS, and 2 mM
L-glutamine. Subconfluent cells ($4x10^6$) were then
20 cultured in DMEM without phenol red, serum or hormones
for an additional 3 days, during which media was changed
twice daily. Cells were incubated for 24 h with or
without hormones (50 nM for E2 and 100 nM for R5020) and
infected with an adenovirus vector containing RIZ1
25 (AdRIZ1; at a concentration of $1x10^{10}$ viral particles/15
cm dish) or the empty vector (AdNull) (He et al., Cancer
Res. 58:4238-4244 (1998)). Cells were then grown for an
additional 48 h in DMEM without phenol red supplemented
with 5 % charcoal-treated FCS (Omega Scientific). Cells
30 were harvested and proceeded to immunoprecipitation and
immunoblot essentially as described (Buyse et al., Proc.
Natl. Acad. Sci. U.S.A. 92:4467-4471 (1995); Liu et al.,
J. Biol. Chem. 272:2984-2991 (1997)). Antibodies used
included RIZ monoclonal antibody 2D7 (Buyse et al., Proc.

Natl. Acad. Sci. U.S.A. 92:4467-4471 (1995)), monoclonal
antibodies versus ERα (sc-8005, Santa Cruz
Biotechnology), progesterone receptor (1A6, DAKO). For
histone methylation analysis, anti-dimethyl-H3-K9 and
5   anti-dimethyl-H3-K4 antibodies (Upstate Biotech) were
used for immunoblot analysis of acid extracted histones
from cells infected with control and AdRIZ1 viruses for
48 hr. For H1 phosphorylation analysis, anti-phosph-H1
and anti-H1 antibody (Upstate Biotech) were used for
10 immunoblot analysis of acid extracted histones.

Mice

RIZ1 -/- mice were generated as described in
Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001).
Briefly, a PR-domain-encoding cDNA fragment of rat RIZ1
15 cDNA was used to screen a mouse 129Sv genomic library
(Stratagene) to obtain mouse genomic DNA. The targeting
construct was designed to insert the PGK-neo^r-BpA cassette
(Soriano et al., Cell 64:693-702 (1991)) into exon 5 and
to place the Herpes Simplex Virus TK cassette from
20 PMC1-TK (Mansour et al., Nature 336:348-352 (1988))
external and adjacent to the region of homology. The
targeting plasmid was linearized by XhoI digestion and
electroporated into D3 ES cells (Gossler et al., Proc.
Natl. Acad. Sci. USA 83:9065-9069 (1986)). The ES clones
25 were selected in media containing G418 (150 µg/ml) and
ganciclovir (2.0 µM). Homologous recombination events
were screened by Southern blot analysis with XbaI
digestion and hybridization to the 0.5-kb XbaI-BglII 5'
probe or with NotI plus EcoRI digestion and hybridization
30 to the 0.5-kb SacII-NotI 3' probe. Five RIZ1+/- ES
cell-lines were microinjected into C57BL/6 blastocysts
that were implanted subsequently into the uteri of Balb/c
foster mothers. Male chimeric mice generated from two
RIZ1+/- ES clones transmitted the RIZ mutation into

germline. The presence of the RIZ1 mutant allele in the F1 animals was confirmed by Southern blotting and polymerase chain reaction (PCR) analysis of genomic DNA from the tail of animals. RIZ1+/- F1 animals were bred

5   together to generate RIZ1-/- mice.

To examine reproductive functions, female RIZ1-/- mice were bred with male RIZ1+/- mice, female RIZ1+/- mice were bred with male RIZ1-/- mice, and breedings between heterozygous mutants were also

10   performed. The number of litters was recorded, and all mice were routinely measured for body weight throughout the studies.

Analysis of gene expression

For RIZ staining, the RIZ rabbit antiserum

15   KG7.1S (Buyse et al., Proc. Natl. Acad. Sci. U.S.A. 92:4467-4471 (1995)) was used at an optimal dilution of 1/200. For ERα staining the polyclonal rabbit anti-ERα antibody (sc-542 Santa Cruz) was used at a dilution of 1/250, and for PR staining the polyclonal rabbit anti-PR

20   antibody (A0098 DAKO) was used at a dilution of 1/100, essentially as described (Tibbetts et al., Biol. Reprod. 59:1143-1152 (1998)). Tissues were fixed in Bouin's solution and processed by routine methods for embeddement in paraffin and sectioning (5 μm). For RIZ and ER

25   staining, sections were treated with trypsin (Zymed), whereas for PR staining, the sections were subjected to treatment using the Target Retrieval Solution (DAKO), all performed according to the manufacture's instructions. Sections were then incubated with the primary antibody

30   followed with a biotinylated secondary antibody. The localization of the primary antibody was visualized with the imidazole-DAB reaction producing a brown colored stain, followed by hematoxyline counterstaining and

routine processing for bright-field microscopy analysis.
Negative controls included omission of the primary
antibodies and incubation with the preimmune KG7.1S
antiserum, whereas colorectal xenograft tumors infected

5   with AdRIZ1 were used as positive control for RIZ
expression (Jiang and Huang, Cancer Res. 61:1796-1798
(2001)).

     For RT-PCR analysis of RIZ gene expression,
tissues from 7-week old RIZ1 +/+ mice were pulverized in

10   liquid nitrogen and total RNA was isolated using the
TRIZOL reagent (Gibco BRL). cDNA was synthesized using
the first strand cDNA synthesis kit (Gibco BRL). Oligos
RP260 (5'-CTC ATT CAT CTA AGA AAG GTG G-3'; SEQ ID NO:16)
+ RP259 (5'-TGA TTC CAG GTC ACT TCA GG-3'; SEQ ID NO:17)

15   and RP170 (5'-GAA GCC AAA GGC CTC TCA TC-3'; SEQ ID
NO:18) + K05 (5'-AGA CTC TGG CTG AGG TAC C-3'; SEQ ID
NO:19) were used in standard PCR conditions encompassing
30 cycles at an annealing temperature of 59°C to amplify
the RIZ1+2 and RIZ1 specific fragments, respectively.

20   *Analysis of target organs in response to hormone
treatments*

     To investigate E2-mediated increments in
proliferation and hyperemia of uteri and changes in the
cellular organization of the vaginal epithelium (Xu et

25   al., Science 279:1922-1925 (1998)), 8-week old female RIZ
-/- and RIZ +/+ mice were ovariectomized (OVXed). At day
15-17 following OVX, mice were treated with S.C.
injections of E2 (0.8 ng/g/day; Sigma) or with the
vehicle (0.1 ml corn oil) alone for three days. The mice

30   were then sacrificed at day 18 and the uterine wet weight
was measured, and vaginal tissue was collected. The
tissues were processed as above and vaginal specimens
were stained with routine H & E and uterine specimens for

PR expression. The thickness of the vaginal epithelium and the cornified layer was photographed and measured as described in Figure 4 using the Spot 3.2.4. software (Diagnostic Instruments).

5   To evaluate the P- and E2-mediated decidual response a previously described protocol was used (Lydon et al., Genes Dev. 9:2266-2278 (1995)); Xu et al., Science 279:1922-1925 (1998)). Briefly, 8 week old female mice were OVXed and at day 10-12 following OVX,
10 mice were treated with S.C. injections of E2 in corn oil (100 ng/day), then treated with P (1 mg/day; Sigma) + E2 (6.7 ng/day) from day 16-23. Six h after the third P + E2 injection on day 18, the left uterine horn (right horn served as a control) was traumatically stimulated by
15 insertion of a burred needle proximal to the cervix and longitudinally scratching the entire length of the uterine horn antimesometrially. The mice were sacrificed on day 23, six hour after the last P + E2 injection, and the wet weights of the left and right uterine horns were
20 measured.

        To measure effects on mammopoiesis by normal development, pregnancy, and female sex hormone treatment the following protocols were used (Xu et al., Proc. Natl. Acad. Sci. USA 97:6379-6384 (2000); Xu et al., Science
25 279:1922-1925 (1998)). To determine the effects of normal pubertal development on mammary gland growth, 7 week old virgin female mice were sacrificed. Additionally, pregnant female mice at day 19 of first pregnancy were sacrificed. To examine the effects of E2
30 + P treatments on mammary gland development, 8 week old female mice were OVXed and at 14 days following OVX treated with S.C. 21-day releasing hormone pellets containing 0.1 mg of E2 and 10 mg P4 or S.C. placebo pellets (Innovative Research of America). On day 35, the

mice were sacrificed and the uterus was wet weighed and
analyzed microscopically to ensure actual hormone
release. In all cases, whole mounts were prepared from
the fourth mammary gland and carmine-stained according to

5 standard procedures(Evans et al., _Oncogene_ 19:989-991
(2000)), and the relationship between mammary ducts and
mammary fat pad, extent of branching as well as number of
branches were investigated microscopically.

In order to evaluate the response to

10 testosterone, twelve week old male mice were
orchiectomized (ORCed) and testicles were wet weighed (Xu
et al., _Science_ 279:1922-1925 (1998)). Nine days
following ORC, mice were treated during days 9-15 with
S.C. injections of testosterone (3 mg/kg/day, Sigma) or

15 vehicle alone (0.1 ml corn oil). On day 16, mice were
sacrificed and the prostate and the pars prostate of the
urethra (for technical reasons) were removed and wet
weighed.

Statistical analysis

20   Student's unpaired t-test was used for
statistical evaluation of means, with $P<0.05$ considered
to be significant. All results are expressed as mean ±
SEM (standard error of the mean) or mean ± SD (standard
deviation, when stated). Fisher's exact test was used

25 for evaluation of the difference between ER-negative and
ER-positive breast cancers, with $P<0.05$ considered to be
significant.

Methylation assays

Methylation reactions (30-40 µl) contained 20

30 mM Tris-HCl (pH8.0), 0.2 M NaCl, 0.4 mM EDTA,
affinity-purified GST-RIZ1 or immunoprecipitation

products, free histones (20-40 µg) purified from acid
extraction of human cancer cells (A549 or U2OS), and 3 µl
(1.65 µCi and 21 pmole)[methyl-^{3}H]-adenosyl-methionine
(Amersham-Pharmacia).   Nuclear extracts were prepared
5   according to standard procedures and used for
immunoprecipitation.   Antisera used for
immunoprecipitation were anti-KG7.1S and preimmune serum
as described previously (Buyse et al., <u>Proc. Natl. Acad.
Sci. U.S.A.</u> 92:4467-4471 (1995)).   Similar HMT activity
10   has also been observed with immunoprecipitates using a
different serum (data not shown), 1715, which was
described previously (Steele-Perkins et al., <u>Genes Dev.</u>
15:2250-2262 (2001)).   Various amount of SAH (Sigma) were
added in some methylation reactions.

15   To determine the methylated residues on H3 and
H1, H3 N terminus 1-20 residue peptides (Upstate
Biotech), non-acetylated and acetylated on K9 and K14,
and H1 N terminus 15-37 and 12-31 peptides were used as
substrates for in vitro methylation reactions as
20   described above.   The reactions were resolved on
Tris-Tricine 10-20% gel (Invitrogen) followed by
fluorography.   The ^{3}H-labelled H3 or H1 peptides were
purified by HPLC and sequenced from the amino termini in
an Applied Biosystems model 477A Protein Sequencer.
25   After conversion, the samples were collected for
determination of radioactivity by scintillation counting.

            Histones from nocodazole treated cells were
isolated by acid extraction of U2OS cells treated for 48
hour with 50 ng/ml nocodazole.   Histones (20 mg) were
30   treated with lambda protein phosphatase (400 U) (New
England Biolabs) for 1 hr at 30 0C in buffer.   Control
histones were treated the same except that phosphatase
was omitted from the incubation mixture.

<u>Results</u>

*RIZ1 is a specific coactivator of ER and progesterone
receptor*

RIZ1 has been shown to be a coactivator of
5   estrogen receptor (ER) (Abbondanza et al., <u>Proc. Natl.</u>
<u>Acad. Sci. USA</u> 97:3130-3135 (2000); Steele-Perkins et
al., <u>Genes Dev.</u> 15:2250-2262 (2001)). To better
understand the coactivator function of RIZ1, the effects
of RIZ1 on ER were compared with its effects on several
10   other NHRs. As shown in Fig. 1A, RIZ1 enhanced ER and
progesterone receptor (PR) function in a ligand- and
dose-dependent manner. In contrast, RIZ1 had no major
effects on the activities of androgen receptor, retinoic
acid receptor a (RARα), retinoic X receptor α (RXRα),
15   thyroid hormone receptor (not shown), glucocoticoid
receptor (not shown), and vitamin D receptor (not shown).
Similar to what has been found with ER (Steele-Perkins et
al., <u>Genes Dev.</u> 15:2250-2262 (2001)), RIZ2 and RIZ1
missense mutants (C106Y and I188V) failed to coactivate
20   progesterone receptor (Fig. 1A). The introduction of a
point mutation in the LXXLL motif of RIZ also produced a
protein unable to act as a coactivator for progesterone
receptor (Fig. 1A). Consistent with RIZ1 effects on ER
and progesterone receptor, protein complexes containing
25   RIZ1 and these receptors were detected by a
coimmunoprecipitation assay (Fig. 1B). Thus, RIZ1 is a
specific coactivator of estrogen receptor and
progesterone receptor but not for other nuclear hormone
receptors, and the intact PR/SET-domain of RIZ1 is
30   essential for its function.

To confirm the expected RIZ expression in the
target organs of E2 and progesterone, RIZ expression was
determined by immunohistochemistry. The gene products

displayed nuclear staining in the uterus, including both
glandular and luminal epithelium as well as stromal and
myometrial cells, similar to the expression of ER
(Fig.1C). Additionally, RIZ is expressed in both mammary

5   and vaginal epithelium. For these experiments, the serum
anti-KG7.1S, which recognizes both RIZ1 and RIZ2, was
used (Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-
4471 (1995); Liu et al., J. Biol. Chem. 272:2984-2991
(1997)). Positive results from this antibody likely

10 reflect expression of both proteins because they are
coexpressed in most tissues (Buyse et al., Proc. Natl.
Acad. Sci. USA. 92:4467-4471 (1995); Liu et al., J. Biol.
Chem. 272:2984-2991 (1997)). Nonetheless, by RT-PCR
analysis using primer sets that can differentiate RIZ1

15 from RIZ2, it was confirmed that RIZ1 was indeed
expressed in various target tissues of female sex steroid
hormones (Fig. 1D).

*Partial hormone resistance in target organs of RIZ1
deficient female mice*

20   The RIZ1 mutant mice are deficient in RIZ1, but
not RIZ2, and therefore lack specifically any PR-domain
related functions of the gene (Steele-Perkins et al.,
Genes Dev. 15:2250-2262 (2001)). These animals are
viable and fertile and show no gross developmental

25 defects (Steele-Perkins et al., supra (2001)). To
determine the physiological role of RIZ1 in steroid
hormone action, uterine growth in response to E2 was
first examined in ovariectomized (OVXed) mice (Lubahn et
al., Proc. Natl. Acad. Sci. USA 90:11162-11166 (1993)).

30 Wild-type or heterozygous RIZ1 mutant mice responded to
E2 treatment with a 5.3 $\pm$ 0.5 and 5.4 $\pm$ 0.4 fold increase
in uterine wet weight. Uteri of homozygous RIZ1 mutants
showed a smaller increase of about 3.7 $\pm$ 0.4 fold
(P<0.001 for +/+ vs. -/-; Fig. 2A and B). Uterine

response to mechanical traumatization (decidual stimulation) is mainly a progesterone receptor-dependent process (Lydon et al., Genes Dev. 9:2266-2278 (1995)). OVXed mice were treated with a high dose of progesterone

5   and a low dose of E2, followed by mechanical stimulation of the left uterine horn of each animal. The unstimulated left uterine horn served as a control. The decidual response resulted in an increase in the uterine horn size and was consistently observed in the stimulated

10   left uterine horn in wild-type mice. Only a partial response was observed in the uterine horn of the RIZ1 null mutant (Fig. 2C). These results suggest that RIZ1 is required for maximal uterine response to E2 and progesterone in vivo.

15   Vaginal cornification in response to a three-day treatment of E2 was examined in OVXed mice. The hormone produces an increased vaginal thickening and cornification of the epithelium (Lubahn et al., supra (1993)), which was consistently seen in the wild-type

20   mice (Fig. 2D,E). RIZ1 null female mice, however, failed to show a maximal such response, thus suggesting that RIZ1 is required for vaginal response to E2 in vivo.

         The epithelial and stromal compartments of the uterus were then analyzed for E2-induced changes in

25   progesterone receptor expression. Such a regulation via ER is highly compartment-specific and mimics the changes in the uterus during the estrous cycle (Tibbetts et al., Biol. Reprod. 59:1143-1152 (1998)). Progesterone receptor staining in OVXed RIZ1 +/+ and -/- mice treated

30   with vehicle was apparently similar and showed strong immunoreactivity in almost all cells of both the luminal (LE) and glandular epithelium (GE), whereas only a fraction of stromal and myometrial cells were positive (Fig. 2G, data not shown). As expected, E2 treatment of

wild type animals had dual effect on progesterone
receptor expression, reducing the levels in the LE and
increasing the levels in the stromal and myometrial
compartments.   Immunoreactivity in the GE was unchanged,

5   consistent with previous findings showing that E2 alone
does not regulate progesterone receptor expression in
this compartment (Fig. 2F,G) (Tibbetts et al., supra
(1998)).   Upon E2 treatment of RIZ1 deficient animals,
the decrease of progesterone receptor levels in the LE

10   was nearly 8-fold less than in wild type tissues, and the
increase of the receptor level in the stroma and
myometrium was nearly 2-fold less than normal.   These
findings suggest that RIZ1 plays a role in the E2
regulation of progesterone receptor expression in the

15   uterus.

         Steroid hormones play an important role in
mammopoiesis (Hennighausen and Robinson, Genes Dev.
12:449-455 (1998)).   Both E2 and progesterone are
essential for alveolar development during pregnancy.   In

20   7-week-old wild-type females, mammary ducts had started
to grow into the mammary fat pad but with no apparent
differences in the age-matched RIZ1 null mutants (Fig. 3A
and B).   However, when stimulated by pregnancy, alveolar
structures in wild-type mammary glands were highly

25   developed and appeared on all ductal sections, filling
the interductal spaces.   In the RIZ1 mutant mammary
glands, alveoli were much less developed in terms of
number and size of alveoli, and fewer alveoli were
observed at the ends of ducts at the same stage of

30   pregnancy (Fig. 3C through F).   Although mammary glands
of RIZ1 null mutants can still produce milk, these
results suggest that RIZ1 is required for normal mammary
ductal elongation and alveolar development in vivo during
pregnancy.   Mammary gland development in response to E2

35   and progesterone treatment in OVXed adult mice was also

examined. E2 and progesterone stimulated a complex ductal arborization and extensive alveolar formation in mammary glands of wild-type mice, which mimics a stage of mammopoiesis in early pregnancy. In the mammary glands

5 of RIZ1 mutant mice, only partial ductal growth was observed after hormone treatment (Fig. 3G through J). Thus, RIZ1 seems to be required for efficient proliferation and differentiation of the mammary gland in response to E2 and progesterone.

10 *Normal steroid hormone response in RIZ1 deficient male mice*

Although RIZ1 is expressed in target tissues of male steroid hormones such as testis (Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995)), it does not

15 appear to serve as coactivator for androgen receptor (Fig. 1A). To study this further, prostate growth was measured in orchiectomized male mice after they were treated with androgen. Similar to female mice, no difference in total body weight was noted in RIZ1 null

20 mutant male animals (Table 1).

Table 1

	Mean ± S.D. (number of mice weighed)		
	5 weeks	8 weeks female	12 weeks female
RIZ1-/-	Female - 16.83 + 1.64(11) 83	21.28 + 2.70(32)	24.27 + 2.21(13)
	Male - 19.24 + 2.25(7)		
RIZ1+/-	Female - 16.83 + 2.01(15)	20.49 + 2.70(12)	24.51 + 3.22(23)
	Male - 20.00 + 2.56(15)		
RIZ1+/+	Female - 18.52 + 1.55 (5)	20.37 + 2.13(31)	21.71 + 3.49(13)
	Male - 20.61 + 2.72(7)		

Eight days after orchiectomization, prostates in both wild-type and mutant animals regressed. Injection of testosterone for 7 days stimulated prostate growth in wild-type animals as well as in RIZ1 null animals. Wild-type and RIZ1 null mutant animals had similar ratios of the weight of prostate and urethra to body weight (1.19 ± 0. 03 vs. 1.14 ± 0.03; n=10 and 9 mice for +/+ and -/-, respectively; p=0.23). They also had similar ratios of testis weight to body weight (7.7 ± 0. 4 vs. 7.4 ± 0.4; n=14 for both genotypes; p=0.58). Thus, tissue responses to testosterone are not significantly affected in mice lacking RIZ1, consistent with the lack of effect of the gene on androgen receptor transactivation functions (Fig. 1A).

Reduced litter size by RIZ1 deficient female mice

The reproductive abilities of RIZ1 mutant mice were assessed. Female RIZ1-/- mice were bred with male RIZ1+/- mice and as controls, female RIZ1+/- mice were bred with male RIZ1-/- mice. Also, breedings between

heterozygous mutants were conducted. The average litter size from breeding RIZ-/- female x RIZ1+/- male was 6.6 ± 3.2 (S.D.; n = 35 litters) but the average litter size was 8.4 ± 2.0 (n = 20 litters) for litters of breeding

5   RIZ1+/- female x RIZ1-/- male (P < 0.01), and 8.0 ± 2.2 (n = 50 litters) for litters of breeding between heterozygous mice (P < 0.02). The results indicated a slightly compromised reproductive ability associated with the female, but not male, mice deficient in RIZ1,

10   consistent with the above results showing defective tissue responses to steroids in female but not male animals.

Regulation of ER-dependent transcription in breast cancer by RIZ1 and RIZ2

15   The above results suggest a physiological role of RIZ1 in E2 response of normal tissues. In view of RIZ1's tumor suppressor role, the question was posed whether RIZ also plays a role in the E2-response of breast cancer cells. RIZ1 is often lost but RIZ2 is

20   uniformly present in breast cancer (He et al., Cancer Res. 58:4238-4244 (1998); Jiang and Huang, Histol. Histopathol. 15:109-117 (2000)). RIZ2 does not serve as a coactivator of ER and can actually neutralize the coactivator function of RIZ1 (Steele-Perkins et al.,

25   Genes Dev. 15:2250-2262 (2001)). Therefore, the effects of overexpressing RIZ1 and RIZ2 in MCF-7 were examined in breast cancer cells that express ER and RIZ1 (He et al., supra (1998)). When cotransfected with the ERE-tk-CAT reporter into MCF-7 cells, RIZ1 enhanced estrogen-induced

30   reporter activity (Fig. 4A). In contrast, E2 induced reporter gene transcription was suppressed by RIZ2 expression. In CV-1 cells that lack ER, reporter gene expression was not affected by RIZ1, RIZ2, and E2 (Fig. 4B). The data suggest that RIZ1 can sensitize breast

cancer to E2 and that decreasing RIZ1 activity, as achieved by RIZ2 expression, can inhibit E2 sensitivity of breast cancer.

RIZ1 gene silencing in hormone resistant breast cancer

5   If RIZ1 appears to facilitate E2-mediated normal and breast cancer growth as indicated by the above studies, why is it also a tumor suppressor and often silenced in breast cancer (He et al., supra (1998))? Upon re-analysis of the published data, it was found that

10   RIZ1 loss is related to hormone resistance (as represented by ER status) among those breast cancer cell lines examined (Table 2).

A methylation-specific PCR assay was developed to examine RIZ1 promoter methylation, which showed that

15   DNA methylation is a good indicator of lost or decreased RIZ1 mRNA expression in tumor cell lines and tissues (Du et al., Cancer Res. 22:8094-8099 (2001)). Methylation of RIZ1 promoter was found to be common in breast cancer tissues, occurring in 11 of 25 breast cancer tissues

20   examined. Except for BT20 and ZR75-1 cell lines, the mRNA expression data in Table 2 are from (He et al., supra (1998)). Expression levels below 10% of normal were scored negative (-).

Table 2

	RIZ1		ER
	Methylation	mRNA	
MB468	+	-	-
MCF-7	-	+	+
MB-435	+	-	-
T47-D	-	+	+
MB231	+	-	-
BT474	-	+	+
SKBR3	+	-	-
BT549	-	+	-
BT20	-	+	-

To address the relationship between RIZ1 and
hormone responsiveness of breast cancer tissues, the ER
expression status was examined for 18 of the 25 samples
for which tissue sections were available. ER expression
was determined by immunohistochemistry (below 10%
positivity was considered negative) and was scored in a
double-blind fashion without knowledge of the methylation
status of these samples. The results showed the same
trend as previously found in cell lines that ER negative
tumors tend to be RIZ1 negative (Table 3).

Table 3

Expression	ER (-)		ER (+)	
	RIZ1 (-)	RIZ1 (+)	RIZ1 (-)	RIZ1 (+)
# tumor cases	83% (5/6)	17% (1/6)	42% (5/12)	58% (7/12)
# cell lines	67% (4/6)	20% (2/6)	0%	100% (4/4)
# Total	75% (9/12)	25% (3/12)	31% (5/16)	
	69% (11/16)			

Thus, 5 of 6 ER-negative cases were RIZ1
negative compared to 5 of 12 ER-positive cases. Together
with data from cell lines, these results shows that there
is a significant association between RIZ1 silencing and
ER-negative breast cancers (RIZ1 silencing found in 9 of
12 ER-negative samples versus 5 of 16 ER-positive
samples, $P < 0.05$). The results also reveal that nearly
half of ER-positive cancer tissues were RIZ1 negative (5
of 12), indicating a potential role of RIZ1 silencing in
the development of hormone resistance of ER-positive
breast cancers. Together, these studies indicate a dual
status of RIZ1 in breast cancer that is linked with the
presence or absence of ER, and in turn, the early or late
stage of the disease. The dual status of the gene
appears consistent with its dual role as a tumor
suppressor and an ER/E2-dependent tumor promoter (see
discussion below).

*Histone methyltransferase activity of RIZ1 and regulation
by estrogen*

The results of animal knock out studies
presented here and previously (Steele-Perkins et al.,
5   <u>supra</u> (2001)), as well as the dual role of RIZ1 in breast
cancer discussed above, suggest a physiological role of
RIZ1 as an ER-coactivator/growth promoter and a tumor
suppressor. Because it is only the PR domain of RIZ1
that is missing in these animals or in tumors, owing to
10   normal expression of RIZ2, it was asked whether RIZ1 has
an activity that may explain its dual function as an
ER-coactivator/growth promoter and tumor suppressor.

Recombinant GST fusion proteins of RIZ1's
N-terminus PR domain region were prepared in bacteria.
15   The recombinant proteins were purified and assayed for
histone methyltransferase activity using histones as
substrates and S-adenosyl-[methyl-^{3}H]-L-methionine as
methyl group donor. Reaction products were separated by
SDS-PAGE and visualized by flurography. Specific
20   labeling of histone H4 was observed in the presence of
purified GST product consisting of residues 1-200 of RIZ1
(Fig. 5A). GST alone or a protein consisting of residues
1-161 lacked HMT activity. Furthermore, missense
mutations found in tumors, C106Y, I188V, and A159V,
25   abolished HMT activity. The results suggest that RIZ1
has HMT activity, which requires the conserved PR-domain
(residues 30-160) as well as the non-conserved residues
C-terminal to the PR-domain (residues 162-200).

It was next asked whether the HMT activity of
30   the PR domain may be modulated by additional sequences of
RIZ1 by testing longer RIZ1 N-terminal fragments made in
bacteria. A GST fusion protein expressing the N-terminal
332-residue peptide showed similar activities to the

N-terminal 200-residue peptide in methylating H4 (Fig.
5B). An attempt to test an even longer peptide (520
residue) in bacteria was not successful because of low
yield of protein. The result suggests that residues
5   200-332 may not intrinsically contribute to HMT activity
but could not exclude other regions of RIZ1 (333-1719)
from playing role in modulating the HMT activity of the
N-terminal 200-residue region.

A domain located at the C-terminus of RIZ1
10   protein (amino acids 1514-1680) was previously defined
that can bind to the PR domain of RIZ1 (Huang et al., _J.
Biol. Chem._ 273:15933-15940 (1998)). It was next asked
whether this domain, termed PBD or PR-binding domain, can
regulate the PR HMT activity. The previously described
15   GST fusion protein, GST-hRIZ1(1514-1680) or now termed
GSTRIZPBD, was examined for its effects on the HMT
activity of GSTRIZ332. Unexpectedly, GSTRIZPBD protein
alone showed HMT activity, specifically on H4, suggesting
that the RIZ PBD domain represents a novel catalytic
20   motif of methyltransferase. A synergistic enhancement
(more than additive effects) of HMT activity was observed
when GSTRIZ322 and GSTRIZPBD were both present in HMT
reactions, as indicated by the increased methylation of
H4 and the methylation of a new substrate with a
25   molecular weight similar to that of H1 (Fig. 5C). This
result suggests that the PR domain HMT activity can be
modulated by other regions of RIZ1, such as the PBD
domain.

Enzyme activity of full-length RIZ1 protein
30   from mammalian cells was next assayed. Human
osteosarcoma U2OS cells, which express little RIZ1 (He et
al., _Cancer Res._ 58:4238-4244 (1998)), were infected with
AdRIZ1 virus or control AdNull virus and nuclear extracts
from virus infected cells were immunoprecipitated with

RIZ serum anti-KG7.1S or preimmune serum.
Immunoprecipitated products by RIZ1 serum from
RIZ1-expressing extracts caused methylation of H1, H3,
and H4 (Fig. 6A). A similar result was also obtained
using a different RIZ1 antiserum, 1715 (data not shown).
As controls, HMT activity was not detected for
immunoprecipitates of preimmune serum or when RIZ1
negative nuclear extracts were used for
immunoprecipitation.

10   The data indicate that RIZ1 protein of
mammalian cells has HMT activity, which is consistent
with the activity of truncated proteins obtained from
bacteria (Fig. 5). However, full length protein appears
to target more histone substrates than truncated proteins
from bacteria, probably because it has sequences or
modifications or both that are missing in bacteria
expressed proteins. The results are consistent with
other regions of RIZ protein regulating PR domain HMT
activity.

20   In view of RIZ1's role in tumor suppression, it
was determined whether S-adenosylhomocysteine (SAH), an
analog inhibitor of SAM-dependent methyltransferases
whose abnormal accumulation is linked with
carcinogenesis, can inhibit RIZ1 enzyme activities. The
effect of SAH on the in vitro methylation of histones by
immunoprecipitated RIZ1 was tested. A methylation assay
was performed in the presence of 0, 0.5 and 5 µM of SAH
and 0.42 µM of 3H-SAM. Histone methylation by RIZ1 was
partially inhibited by 0.5 µM of SAH and completely
inhibited by 5 µM of SAH (Fig. 6B). The results show
that SAH is an inhibitor of RIZ1 methyltransferase.

          The methylated lysine residues at position 4
and 9 of histone H3 are the best-characterized histone

lysine methylations with regard to their biological
function. Methylation of H3-K9 is associated with gene
repression and heterochromatin formation, whereas
methylation of H3-K4 is often linked to gene activation
5   (Noma et al., Science 293:1150-1155 (2001); Rea et al.,
Nature 406:593-599 (2000); Strahl et al., Curr. Biol.
11:1-5 (2001)). Each of the two lysine residues can be
targeted by several different SET domain HMTs, which are
either K4 or K9 specific but not both, and often have
10 non-H3 substrates such as H1 (Nishioka et al., Genes &
Dev. 16:479-489 (2002); Rea et al., Nature 406:593-599
(2000); Tachibana et al., J. Biol. Chem. 276:25309-25317
(2001); Wang et al., Mol. Cell. 8:1207-1217 (2001a)). To
determine whether RIZ1 may belong to such a group of
15 H3-K4 or H3-K9 specific HMTs may immediately explain how
RIZ1 functions in transcription. Therefore, it was
determined whether RIZ1 may have any activity toward
H3-K4 or H3-K9. First, it was determined whether RIZ1
can alter the methylation of these residues in vivo in
20 mammalian cells. Histones were extracted from U2OS cells
infected with AdRIZ1 or control virus and analyzed by
western blot analysis using antibodies that recognize
specifically H3-K9 methylation or H3-K4 methylation. The
result shows that RIZ1 increased H3-K9 methylation but
25 did not affect H3-K4 methylation (Fig. 6C), consistent
with H3-K9 but not H3-K4 as a potential target of RIZ1
methyltransferase.

        To show that RIZ1 can directly methylate H3-K9,
peptide methylation and sequencing experiments were
30 performed. A peptide consisting the N-terminal 1-20
residues of H3 was indeed methylated in vitro by
immunoprecipitated RIZ1 protein using
S-adenosyl-[methyl-3H]-L-methionine as methyl donor (Fig.
6D). In contrast, a peptide containing acetylated K9 and
35 K14 was not methylated, consistent with K9 but not K4 as

a target of RIZ1. The migration of the peptide as a
doublet has also been observed by others, and may be due
to the poor resolution of SDS-PAGE. The methylated
peptide was next purified by HPLC and subjected to

5   peptide sequencing analysis. Purified peptides were
sequenced from the amino termini and the radioactivity of
each of the sequenced residues was determined by
scintillation counting. A single radioactive peak at
lysine 9 was observed (Fig. 6E). The results suggest

10   that H3-K9, but not H3-K4, is a target of RIZ1
methyltransferase.

Next, the possible methylation site on H1 was
determined. A H1 peptide (aa 15-37) was methylated by
RIZ1 in vitro, which was then subjected to HPLC

15   purification and sequencing analysis. Repeated
·methylation and sequencing analysis showed that lysine 25
was methylated by RIZ1 (Fig. 6F).

The capacity to methylate H3 in a K9 specific
manner suggests a role for RIZ1 in transcriptional

20   repression, which appears to be in conflict with RIZ1's
role as an ER coactivator. To help understand this
apparent paradox, the estrogen effects on RIZ1 HMT
activity were examined. Nuclear extracts from control
and E2 treated MCF-7 cells were immunoprecipitated by

25   RIZ1 serum anti-KG7.1S or preimmune serum. As expected,
HMT activity (labeling of H3 and H1) was detected in the
immunoprecipitates of RIZ1 serum but not preimmune serum
(Fig. 7A, lanes 1 and 3). Interestingly, estrogen
treatment decreased H3 methylation but maintained or

30   slightly enhanced H1 methylation by RIZ1 (Fig. 7A,
lane 4). RNase protection analysis did not reveal
altered RIZ1 or RIZ2 gene expression by E2 treatment of
MCF-7 cells (not shown). Furthermore, E2 also similarly
regulated the HMT activity of overexpressed RIZ1 protein

in AdRIZ1 virus infected MCF-7 cells (Fig. 7A, lanes
5-6); cells infected with control virus showed similar
results as non-infected cells (not shown). Thus, E2
appears to regulate RIZ1 HMT activity in a selective way,
5   inhibiting specifically methylation of H3 but not H1.
The data suggest that E2 may turn RIZ1, possibly with a
dual role of transcriptional repressor and activator,
into a transcriptional activator by selectively turn off
its repressor activity as represented by H3 methylation
10   while enhancing its putative activator activity,
presumably represented by H1 methylation.

Although the function of H1 methylation remains
to be characterized, the results here suggest, at least,
that RIZ1 methylation of H1 is unlikely to be similar to
15   H3-K9 methylation, in view of the contrasting behavior of
these two methylation activities of RIZ1. Because H1
phosphorylation has been linked with transcriptional
activation (Bhattacharjee et al., Mol. Cell. Biol.
21:5417-5425 (2001); Dou and Gorovsky, Mol. Cell. 6:225-
20   231 (2000)), it was next asked whether E2 or RIZ1 can
enhance H1 phosphorylation. Histones were extracted from
MCF7 cells infected with AdRIZ1 or control virus and
analyzed by western blot analysis using antibodies that
recognize specifically phosphorylated H1. The result
25   shows that both E2 and RIZ1 increased H1 phosphorylation
(Fig. 7B), suggesting that E2 or RIZ1 may function to
stimulate transcription in part by mediating enhanced H1
phosphorylation. That RIZ1 methylation of H1 was
positively correlated with H1-phosphorylation suggests
30   that such methylation is correlated with transcriptional
activation.

Next, the methyl acceptor potential of
phosphorylated H1 with non- or hypophosphorylated H1 was
compared. Histones were isolated from cells arrested at

G2/M by nocodazole, which were enriched for
phosphorylated H1, and used as substrates for in vitro
methylation by RIZ1. H1 from nocodazole treated cells
appeared to serve as a poor substrates compared to H1

5   from control cells (not shown). This result indicates
either that H1 isolated from nocodazole treated cells was
both phosphorylated and methylated or that
phosphorylation of H1 may prevent its methylation. To
resolve this, histones were treated with phosphatase

10   prior to use as methylation substrates. Phosphatase
treatment removed a significant amount of phosphate on H1
but did not affect its ability to serve as methylation
substrates (not shown). Together, these results suggest
that phosphorylated H1 is associated with a higher extent

15   of methylation than non- or hypophosphorylated H1 and
that methylated H1 is associated with a higher degree of
phosphorylation than non- or hypomethylated H1.

        The time course response of RIZ1 methylation
activity to E2 treatment was then studied. A decrease of

20   RIZ1 H3 methylation activity was found at 4 hr post E2
treatment, which stayed at low levels thereafter (not
shown). Because E2-induced cell proliferation is not
known to occur prior to at least 8 hr of treatment, the
data suggest that inhibition of RIZ1 H3-methylation

25   activity by E2 is unlikely a result of cell
proliferation.

        The observation that E2, a growth factor, has a
sustained inhibitory effect on RIZ1 methylation of H3 but
not H1 suggests that H3 and H1 methylation activity of

30   RIZ1 may be oppositely linked with cell growth. To
further study the contrasting behavior of H1 and H3
methylation, regulation of RIZ1 HMT activity by cell
growth was examined, using
12-O-tetradecanoyl-phorbol-13-acetate (TPA) treatment of

U937 myeloid leukemic cells as a model system. U937
cells undergo growth arrest and terminal differentiation
into monocytes when exposed to TPA. This cell model was
selected for study because it has been shown in these
5   cells that RIZ gene expression is only marginally
regulated by TPA. A slight increase (less than 2 fold)
in RIZ1 mRNA, but no change in RIZ2 levels, was not
observed until after 3 or more days of TPA treatment
(Gazzerro et al., Mol. Med. 7:552-560 (2001)). Thus, any
10   significant changes in HMT activity, especially a
decrease, may not be attributed to changes in RIZ1
protein amounts, at least within the first three days of
treatment. The TPA effects on U937 cell growth and
differentiation were confirmed by counting cell numbers
15   and examining cell morphology (not shown). It was also
confirmed that RIZ gene expression was not significantly
altered within the first 3 days of TPA treatment. By
assaying the HMT activity of RIZ1 immunoprecipitates from
nuclear extracts, it was found that H3 methylation was
20   increased, whereas H1 methylation was slightly decreased
at 24 hr following TPA treatment (not shown). Similar
results were observed at 3 days or 7 day spost TPA
treatment, although H3 methylation activity was noted to
be lower than that at 1 day post treatment. No
25   significant change in HMT activity was noted for any
treatment periods of less than 24 hr. The change at 24
hr post TPA treatment was well before any significant
growth arrest had taken place, suggesting that such a
change is unlikely a result of growth arrest. The
30   results suggest that the H3 methylation activity of RIZ1
is linked with growth arrest, whereas its H1 methylation
activity is associated with cell proliferation,
confirming the above described contrasting behavior of
these activities in response to E2.

<u>Discussion</u>

       The above results show that female mice
deficient in RIZ1 display an impaired response to E2 and
progesterone, and reduced reproductive abilities. RIZ1
5   shows coactivator activity for ER and progesterone
receptor but no or weak activity for other NHRs examined.
Furthermore, the results suggest that a coactivator
(RIZ1) may serve as both an E2-dependent growth promoter
in early stage breast cancer and a tumor suppressor in
10 preventing breast cancer progression to an estrogen
independent state. Estrogen mediated transactivation in
breast cancer cells is suppressed by a RIZ1 antagonist
(RIZ2), and hormone resistant breast cancers more
commonly carry a silenced RIZ1 gene than hormone
15 sensitive breast cancers. These data suggest that RIZ1
has HMT activity, which is mediated by two independent
but interacting catalytic motifs. The HMT activity of
the PR domain is linked to both its ER-coactivator
function and tumor suppressor function.
20 Cancer-associated mutations in the PR-domain, which
inactivate its coactivator function, impair the HMT
activity. Also, the HMT activity of RIZ1 can be
regulated by E2, in a fashion consistent with RIZ1's role
as an ER-coactivator.

25   The specificity of RIZ1 in its interaction with
female sex NHRs contrasts to members of the SRC-1/p160
family members, which seem more promiscuous in this
respect. Thus, one may expect more subtle phenotypes for
RIZ1 deficient mice versus SRC-deficient animals.
30 Indeed, this is the case even when comparing to mice
deficient in SRC-1, which shows the mildest phenotypes
among animals deficient in a SRC member (Wang et al.,
<u>Proc. Natl. Acad. Sci. USA</u> 97:13549-13554 (2000); Xu et
al., Proc. Natl. Acad. Sci. USA 97:6379-6384 (2000); Xu

et al., Science 279:1922-1925 (1998)). While SRC-1 and
RIZ1 deficient female animals show similar reduced
sensitivity to female sex hormones, SRC-1 mutation,
unlike RIZ1, also affects mammary duct growth during
5   puberty in virgin mice and hormone response in males.
The partial hormone resistance phenotypes observed in
RIZ1 or SRC-1 deficient mice likely reflect overlapping
functions of RIZ1 or SRC-1 related proteins, for which
there are many. Or, it may be related to an additive
10   effect of RIZ1 and SRC-1 on ER function. Reduced H3-K9
methylation, as induced by E2 inhibition of RIZ1, would,
in and of itself, facilitate transcription activation by
reducing the binding of the heterochromatin protein HP1
to methylated K9 on H3 (Bannister et al., Nature 410:120-
15   124 (2001); Lachner et al., Nature 410:116-120 (2001)).
In addition, transcription would be further enhanced by
the acetylation of the methyl-free H3-K9 by SRC-1 or its
related HATs.

       The partial reduction in tissue response to
20   steroids in RIZ1 deficient mice was accompanied by a
corresponding reduction in target gene expression. E2
induction of progesterone receptor gene expression in the
stroma and myometrium compartments of the uterus was
reduced in RIZ1 deficient mice (Fig 2F-G). The result
25   suggests a physiological role of RIZ1 in facilitating
ER-transcriptional activation function and that such a
coactivator function is likely responsible for the
observed tissue response to E2.

       While the overall tissue response to steroids
30   is only partially reduced by RIZ1 deficiency, this does
not necessarily indicate that RIZ1 is unable to serve a
unique and non-redundant function in facilitating NHR
function. It is possible that at the molecular level,
RIZ1 may be uniquely involved in controlling expression

of certain target genes.   Indeed, estrogen repressed
progesterone receptor gene expression nearly 20-fold in
the luminal epithelial cells of the uterus in the
presence of RIZ1 but only 2-fold in its absence (Fig.

5   2F-G).   Although ER/E2 appear to largely use RIZ1 as a
coactivator, this result further suggests that it can
also employ it as a corepressor in certain circumstances.
It is possible that ER/E2 may enhance RIZ1 methylation of
H3-K9 in certain cellular contexts.

10   That RIZ1 appears required for E2 induced
tissue growth contrasts with its tumor suppressor role.
To help understand the apparent paradox, the role of RIZ1
in breast cancer was studied.   RIZ1 overexpression
enhanced ER-dependent transactivation of reporter gene

15   whereas overexpressing a RIZ1 antagonist, RIZ2, inhibited
the transactivation function of endogenous ER in breast
cancer cells.   Thus, RIZ1 inactivation maybe associated
with decreased hormone response in breast cancers.
Consistently, RIZ1 silencing was shown to be

20   significantly more common in E2 resistant or ER-negative
comparing to ER-positive breast cancers.   It is logical
to expect that alteration of ER coactivators may play a
role in the hormone response phenotypes of breast
cancers.   This idea has been pursued by several previous

25   studies, which found overexpression of SRC3 (AIB1) and
SRA in mostly ER-positive breast cancers (Anzick et al.,
Science 277:965-968 (1997); Kurebayashi et al., Clin.
Cancer Res. 6:512-518 (2000); Murphy et al., Cancer Res.
60:6266-6271 (2000); Thenot et al., Mol. Cell.

30   Endocrinol. 156:85-93 (1999)).   As might be anticipated,
there is a quantitative relationship between ER
concentration in breast cancer tissue and response to E2
(Allegra, Semin. Oncol. 10:23-28 (1983); Osborne et al.,
Cancer 46:2884-2888 (1980)).   The higher the ER amount,

35   the more likely a response.   ER is rarely mutated and the

term ER-negative refers to ER level that is below an empirically determined level. Partial hormone response still exists in such cells with low ER levels. It is therefore expected that loss in one of the coactivators

5   (RIZ1) may further reduce hormone response of such cells. Similarly, silencing of a coactivator in ER-positive breast cancers, as shown here for RIZ1, may play a role in the acquisition of hormone resistance phenotypes of these tumors.

10   For hormone sensitive early stage breast cancer, the dual role of RIZ1 as a tumor suppressor and an ER-coactivator/growth promoter raises the issue whether disabling RIZ1 could be beneficial or not to tumor cell proliferation. It appears that retaining RIZ1

15   may be more favorable than disabling it, in view of the reduction of E2-induced tissue growth in RIZ1 deficient mice. This view is also consistent with the finding that RIZ1 silencing was less common in ER-positive versus ER-negative breast cancers.

20   If RIZ1 may confer growth advantage to early stage breast cancers, why is it not so for late stage ER-negative breast cancers, where RIZ1 is commonly silenced? This is expected for a tumor suppressor that also has an ER-dependent growth promoter function. In

25   breast cancers with functional ER, which, upon E2-binding, may turn RIZ1 from a default tumor suppressor into a coactivator/tumor promoter, RIZ1 is often present and may play a role in promoting tumor growth in the presence of E2. In these tumors, RIZ1 may also play a

30   role in keeping tumor growth in check when E2 is absent. In contrast, in ER-negative breast cancers, where a coactivator would be no longer useful and a tumor suppressor would be undesirable, RIZ1 is frequently lost,

which may play a role in promoting the E2-independent growth phenotype of these tumors.

The questions arises as to why most other ER-coactivators are not silenced in hormone resistant

5   breast cancers (Anzick et al., Science 277:965-968 (1997); Kurebayashi et al., Clin. Cancer Res. 6:512-518 (2000); Murphy et al., Cancer Res. 60:6266-6271 (2000); Thenot et al., Mol. Cell. Endocrinol. 156:85-93 (1999)). This is probably because they are not tumor suppressors.

10 It is likely that only a coactivator with tumor suppressor function would represent a preferred target of inactivation during the transition from hormone sensitive to insensitive tumors. During such transition, hormone sensitive tumors will develop mechanisms to grow in the

15 absence of hormonal stimuli or ER function. In these tumor cells that have survived compromised ER, a coactivator may not be useful anymore, and thus there will be no longer a selective pressure to keep it active. Meanwhile, in the absence of the ER switch, the default

20 tumor suppressor function of a coactivator would be on, which would not be favorable to tumors and a selective pressure to inactivate the coactivator may emerge.

These results suggest that RIZ1 possesses intrinsic HMT activity. The RIZ1 specific region (1-200

25 aa) containing the PR-domain, which is absent in RIZ2, is sufficient for histone H4 methylation in vitro. The PR-domain (residues 30-160) is necessary but insufficient, and requires the less conserved regions (residues 161-200) C-terminal to the PR-domain for HMT

30 activity. The requirement for the region nearby the PR-domain is similar to what has been described for the SET-domain (Rea et al., Nature 406:593-599 (2000)). The activity of the PR domain was impaired by tumor

associated point mutations, consistent with an important
role of the activity in tumor suppression functions.

A new methyltransferase catalytic motif was
identified, the PBD domain, located at the RIZ1
5   C-terminus. This domain can methylate H4 in vitro and
can physically/functionally cooperate with the PR domain
to produce higher HMT activity as well as to broaden
substrate specificity. The PBD domain appears not to be
significantly related to any proteins in the database.
10   That this domain has HMT activity and can modulate PR
activity is consistent with a potential role of this
domain in tumor suppression, as indicated by its deletion
owing to frameshift mutations in microsatellite unstable
cancers.

15   These data show that a bacteria-expressed
200-residue peptide consisting of the PR-domain region of
RIZ1 N-terminus methylated histone H4 while the
full-length protein also used H1 and H3 as substrates.
The result is consistent with a need for other regions of
20   RIZ1, such as the PBD domain, in modulating the PR domain
activity. Indeed, a novel H1 like-protein was methylated
only in the presence of both PR and PBD but not by either
alone. Alternatively, immunoprecipitated RIZ1 may not be
free of RIZ1 bound proteins that could modulate its
25   enzyme activity. RIZ1 may exist as part of a protein
complex and the intact nature of such a complex may be
required by HMT activity. An example of a HMT complex is
the SUV39H1 complex of 650 kDa (Nishioka et al., Genes &
Dev. 16:479-489 (2002)). Finally, mammalian specific
30   post-translational modifications of RIZ1 could play a
role in substrate specific methylation activities. The
latter two possibilities are consistent with the
substrate specific regulation of RIZ1 HMT activity by E2

and TPA, and should be an important topic for future
investigations.

            The results also show that lysine 9 of histone
H3 is a major methylation target of RIZ1 protein. RIZ1
5   protein caused enhanced H3-K9 methylation in vivo and
also methylated H3-K9 in vitro. H3-K9 methylation is
linked with heterochromatin formation and gene silencing,
which in turn, implicates a similar role for RIZ1.
Histone H1 and H4 are also methylated by RIZ1 but the
10 role of H1 and H4 methylations in gene transcription
remains to be understood. A RIZ1 target residue was
identified on H1 in vitro as lysine 25, which is
consistent with the known methylation of H1-K25 in vivo
(Ohe et al., J. Biochem. (Tokyo) 100:359-368 (1986)).

15   That E2 can maintain or enhance methylation of
one substrate (H1) while decrease methylation of another
(H3) suggests a complex mechanism of regulation of RIZ1
activity. The changes in H3 and H1 presumably together
contribute to the ligand-dependent RIZ1 activation of ER
20 transactivation functions. That H3-K9 methylation by
RIZ1 is inhibited by E2 is consistent with RIZ1 as a
coactivator, given that such methylation is linked with
gene silencing. The contrasting change in H1 methylation
suggests that the role of such methylation is likely to
25 be at least different from, if not the opposite of, that
of H3-K9 methylation. H1 phosphorylation is known to be
important in transcriptional activation (Bhattacharjee et
al., Mol. Cell. Biol. 21:5417-5425 (2001); Dou and
Gorovsky, Mol. Cell. 6:225-231 (2000)), although the role
30 of its methylation remains unknown. The finding here
that RIZ1 or E2 induced both phosphorylation and
methylation of H1 suggests a role for H1 methylation
similar to that of H1 phosphorylation. In view of the
coactivator function and the previously described

transcriptional repressor function (Xie et al., <u>J. Biol. Chem.</u> 272:26360-26366 (1997)), RIZ1 appears to have a dual role in both transcription activation and repression. The dual role may be mediated in part by

5　methylation of H3-K9 and H1-K25, respectively (Table 4), which may be turned more toward one direction or the other depending on the protein and promoter environment of RIZ1 at particular circumstances. When RIZ1 is recruited by estrogen to activate transcription, the H3

10　methylation function of RIZ1 would be inhibited and the H1 methylation would be maintained or enhanced. The results suggest a model that RIZ1 has a dual role of a tumor suppressor and an ER-coactivator/growth promoter and the same PR-domain is required for both. ER/E2

15　appears to be a switch that can turn RIZ1 from a tumor suppressor into a growth promoter. The dual role of RIZ1 may be mediated by different substrate usage. The H3-K9 methylation/gene repression function of RIZ1 appears to be associated with growth arrest whereas the H1

20　methylation is associated with cell growth, as indicated by the changes of these methylation activities of RIZ1 during terminal differentiation of U937 cells (summarized in Table 4). The tumor suppressor role of RIZ1 is non-tissue specific but the ER-coactivator function is

25　restricted to ER-positive tissues.

Table 4

RIZ1 function[a]	E2 target tissues or ER(+) breast cancer		Non-E2 target tissues or ER(-) breast cancer	Cell proliferation potential	
	+E2 -			high	low
	E2				
H1-K25 methylation H1 phosphorylation Gene activation Growth promotion	++ +		+	++	+
H3-K9 methylation Gene repression Growth suppression	+ ++		++	+	++

a   The functional activity of RIZ1 is indicated as relatively low(+) or high(++)

It seems paradoxical that E2 employs a tumor
suppressor for its growth promoting functions. That RIZ1
HMT activity is sensitive to E2 raises the possibility
that RIZ1 may have a role as a sensor and breaker to
prevent abnormal hormone-independent growth of cells.
The presence of RIZ1 in early stage breast cancers may
explain the lack of tumor growth in the absence of E2,
while its common absence in advanced breast cancers may
explain the hormone-independent tumor growth. E2 may
serve as a switch to turn off the tumor suppressor
function of RIZ1, perhaps by changing target genes and/or
HMT activities. Given that the HMT function is required
for both tumor suppressor and coactivator functions of
RIZ1, E2 must not simply turn off all HMT activities. It
is therefore reassuring that E2 can selectively inhibit

RIZ1 HMT activity toward one substrate (H3) while
maintain or enhance the activity toward the other (H1).

        In summary, the studies described above
demonstrate that a HMT (RIZ1) is a target of estrogen and
5   is required for efficient female sex hormone action in
vivo. Compromised HMT function of RIZ1 is associated
with estrogen resistant breast cancers, and may prove
important in the transition to hormone resistance and
hormone independent growth during breast cancer
10 progression. By analogy, altered HMT function may also
be involved in clinical syndromes characterized by an
impaired female sex hormone homeostasis such as
osteoporosis, cardiovascular disease, and Alzheimer's
disease.

15   EXAMPLE II

        This example shows various assays suitable for
identifying RIZ1 modulatory compounds and compounds that
modulate progesterone receptor activity.

A.   *Biochemical assay based on binding of RIZ1 to*
20 *universal inhibitors of protein methyltransferase*
activity.

        The best presently known universal inhibitor of
PMT is S-Adenosyl-L- or D-homocysteine (SAH, Ki=5 to 12
µM). A less potent inhibitor is Adenosyl-L-ethionine
25 (Ki=170 µM). SAH mimics the structure of
S-adenosyl-L-methionine (SAM) that is the substrate of
PMT and the methylgroup donor. RIZ1 is expected to bind
to SAH through its PR/PMT motif and its PBD domain.

        Multi-well plates are coated with SAH and each
30 well contacted with compounds from a small molecule

chemical compound library. The plates are incubated with
purified recombinant protein containing the RIZ1 PR/PMT
motif (about 200 amino acids) that has been labeled with
a fluorescent probe. After washing away unbound RIZ1
5   protein, the plates are read by a fluorescence detector.

B. *Biochemical assay based on binding of RIZ1 to ER or PR*

        The coactivator function of RIZ1 on ER and PR
is dependent upon physical complex formation. Binding of
RIZ1 to ER and PR is mediated by the LXXLL motif of RIZ1
10   and is hormone dependent.

        Recombinant RIZ1 protein is coated onto
multiwell plates and each well contacted with compounds
from a small molecule chemical compound library, as well
as estrogen or progesterone. The plates are incubated
15   with purified recombinant ER or PR protein that is
labeled with a fluorescent probe. After washing away
unbound ER or PR protein, the plates are read by a
fluorescence detector.

C.   *Cellular assay based on RIZ1 coactivation of ER or PR*

20   Multiwell plates are seeded with CV1 cells and
the cells transfected with a plasmid containing a ER
responsive promoter linked to a luciferase reporter, a
plasmid expressing ER, and a plasmid expressing RIZ1.
Each well is contacted with compounds from a small
25   molecule chemical compound library. Two days following
transfection, the luciferase activity of each well is
assayed and read by a fluorescence detector.

D. *Assay to determine or verify the in vivo efficacy of modulators of RIZ1*

Once target compounds are obtained through *in vitro* biochemical or cellular high throughput screening,
5 the compounds are tested for *in vivo* efficacy in inhibiting/enhancing the physiological function of RIZ1. Female mice lacking RIZ1 PR/PMT function show decreased response to estrogen and progesterone. Therefore, inhibitors of RIZ1 PR/PMT are expected to decrease
10 estrogen and progesterone responses in mice, while activators of RIZ1 may enhance responses.

Overectomized female mice are treated with estrogen and target compounds for three days. The animals are sacrificed and examined for uterine wet
15 weight and vaginal cornification. In additon, mammay gland growth in response to estrogen and progesterone is determined. 8 week old female mice are ovariectomized at day 0 and treated with E2 (50 µg/day) + P4 (1 µg/day) from day 14-34 (or 21-day releasing hormone pellets
20 containing 0.1 mg of E2 and 10 mg P4; Innovative Research of America). Target compounds are applied together with the hormones. On day 35, the mice are sacrificed and whole mounts prepared and stained. RIZ1 null mutant mice are similarly treated to serve as specificity control for
25 the effects of the target compounds.

All journal article, reference and patent citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference in their entirety.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention.

What is claimed is:

1.   A method of screening for a compound that
modulates RIZ histone methyltransferase activity,
comprising:

5   a) contacting a RIZ or RIZ fragment having histone
methyltransferase activity with one or more candidate
compounds; and

    b) determining histone methyltransferase activity of
said contacted RIZ or RIZ fragment,

10   wherein a compound that modulates RIZ histone
methyltransferase activity is identified.

2.   The method of claim 1, wherein said RIZ is RIZ1.

3.   The method of claim 1, wherein said RIZ is RIZ2.

4.   The method of claim 1, wherein said RIZ fragment
15   comprises amino acids 1-200 of human RIZ1.

5.   The method of claim 1, wherein said RIZ fragment
comprises amino acids 1-332 of human RIZ1.

6.   The method of claim 1, wherein said RIZ fragment
comprises amino acids 1514-1680 of human RIZ1.

20   7.   The method of claim 1, wherein histone H1
methyltransferase activity is determined.

8.   The method of claim 1, wherein histone H3
methyltransferase activity is determined.

9.   The method of claim 1, wherein histone H4
25   methyltransferase activity is determined.

10. A method of screening for a compound that modulates progesterone receptor activity, comprising:

a) providing a RIZ1 modulatory compound;

b) determining the ability of said RIZ1 modulatory

5 compound to modulate progesterone receptor activity, wherein a compound that modulates progesterone receptor activity is identified.

11. The method of claim 10, wherein step a) comprises:

10 i) contacting RIZ1 with one or more candidate compounds;

ii) determining the ability of said candidate compound to selectively bind RIZ1; and

iii) providing a compound from step ii) that

15 selectively binds RIZ1.

12. The method of claim 10, wherein step a) comprises:

i) contacting RIZ1 with one or more candidate compounds under conditions wherein RIZ1 selectively binds

20 a RIZ1-binding partner;

ii) determining the ability of said candidate compound to modulate said selective binding between RIZ1 and said RIZ1-binding partner; and

iii) providing a compound from step ii) that

25 modulates said selective binding.

13.   The method of claim 10, wherein step a)
comprises:

   i)   contacting RIZ1 with one or more candidate
compounds under conditions wherein RIZ1 exhibits a
5 functional activity;

   ii) determining the ability of said candidate
compound to modulate said functional activity; and

   iii) providing a compound from step ii) that
modulates said functional activity.

10   14.   The method of claim 13, wherein said functional
activity is histone methyltransferase activity.

      15.   The method of claim 10, wherein step b)
comprises determining the ability of said RIZ1 modulatory
compound to modulate progesterone receptor-mediated
15 transcription.

      16.   The method of claim 10, wherein step b)
comprises determining the ability of said RIZ1 modulatory
compound to modulate progesterone-dependent mammopoiesis.

      17.   The method of claim 10, wherein step b)
20 comprises determining the ability of said RIZ1 modulatory
compound to modulate progesterone-dependent uterine
development.

      18.   A method of identifying an individual with an
estrogen receptor positive (ER+) tumor having a reduced
25 likelihood of responding to endocrine therapy, comprising
determining the RIZ1 status of said tumor, wherein an
abnormal RIZ1 status identifies said individual as an
individual with a reduced likelihood of responding to
endocrine therapy.

19.   The method of claim 18, wherein abnormal RIZ1 status is evidenced by RIZ1 promoter methylation.

20.   The method of claim 18, wherein abnormal RIZ1 status is evidenced by decreased RIZ1 protein expression.

5   21.   The method of claim 18, wherein abnormal RIZ1 status is evidenced by decreased RIZ1 mRNA expression.

22.   The method of claim 18, wherein abnormal RIZ1 status is evidenced by a mutation in the RIZ1 gene.

23.   The method of claim 18, wherein abnormal RIZ1
10   status is evidenced by altered RIZ1 histone methyltransferase activity.

24.   The method of claim 23, wherein said altered RIZ1 histone methyltransferase activity is increased H1 methylation or decreased H3 methylation.
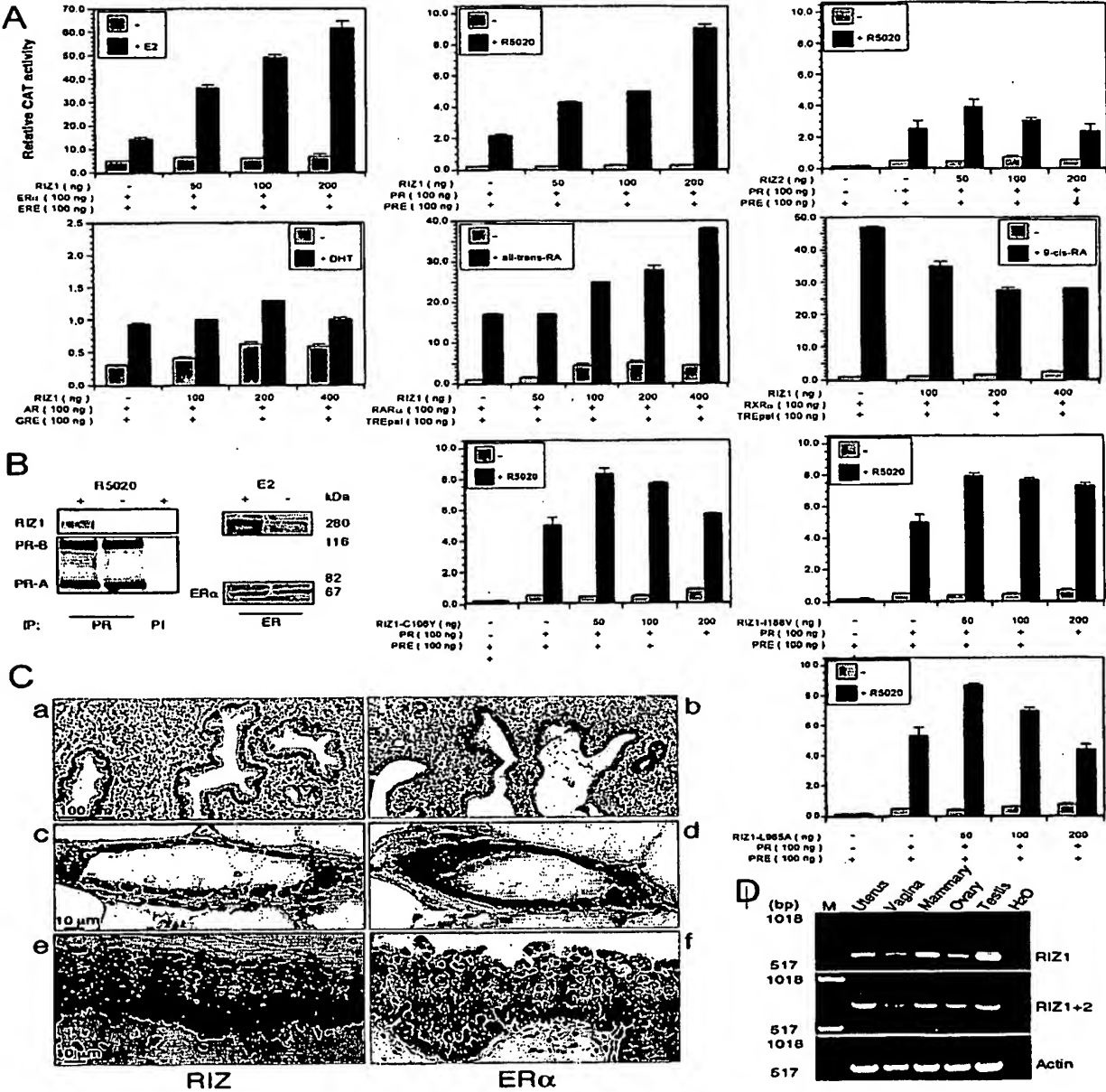
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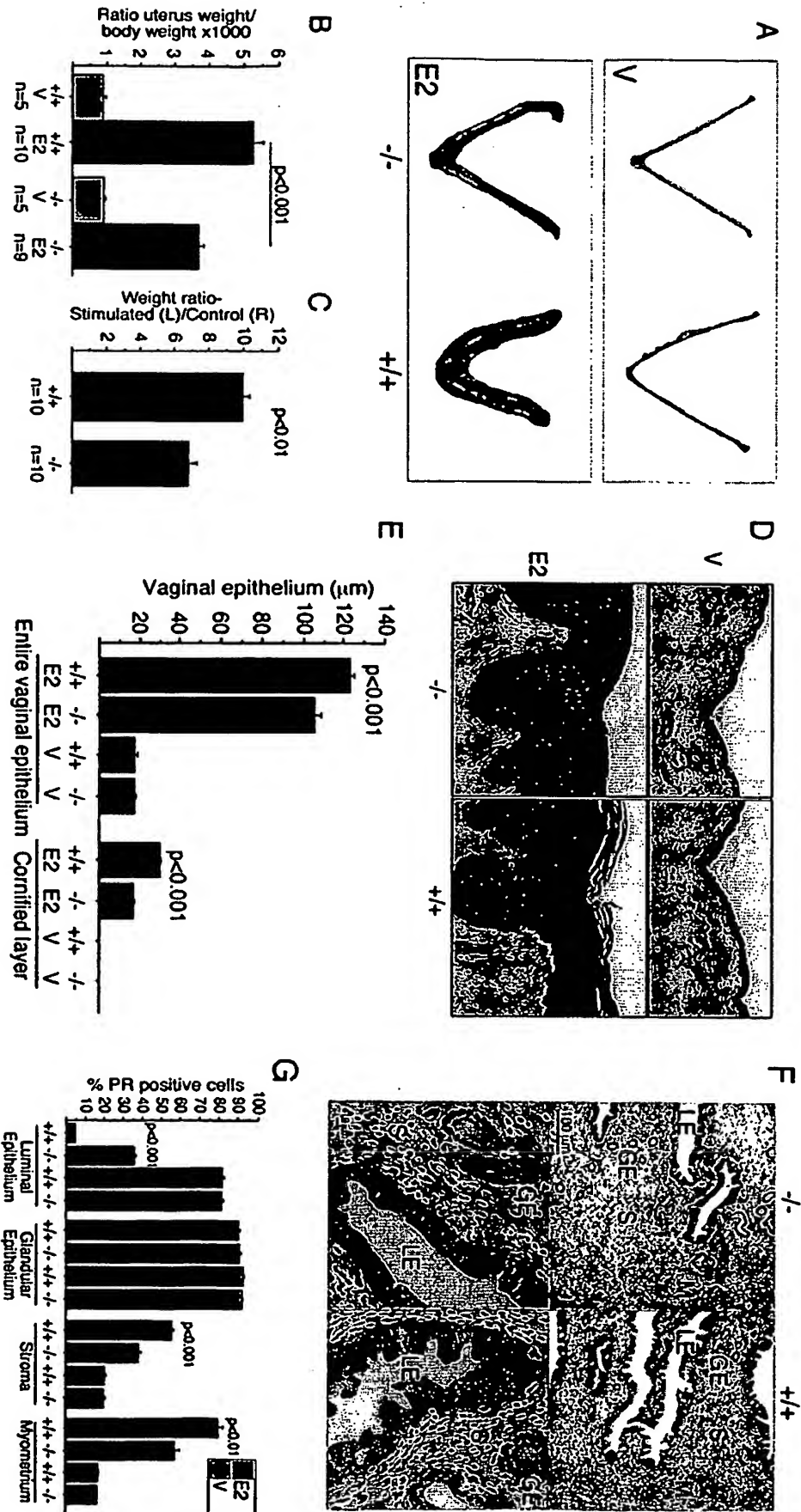
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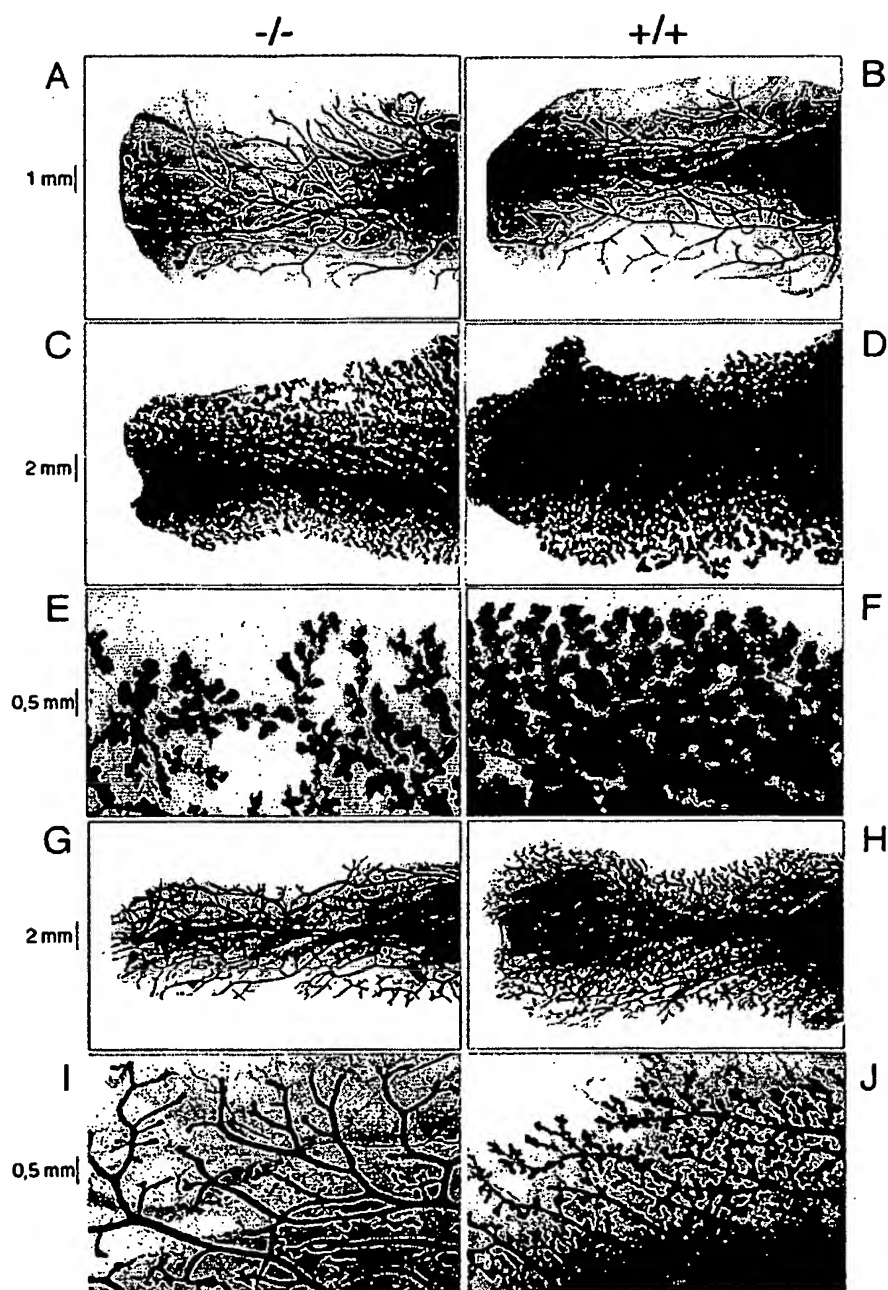
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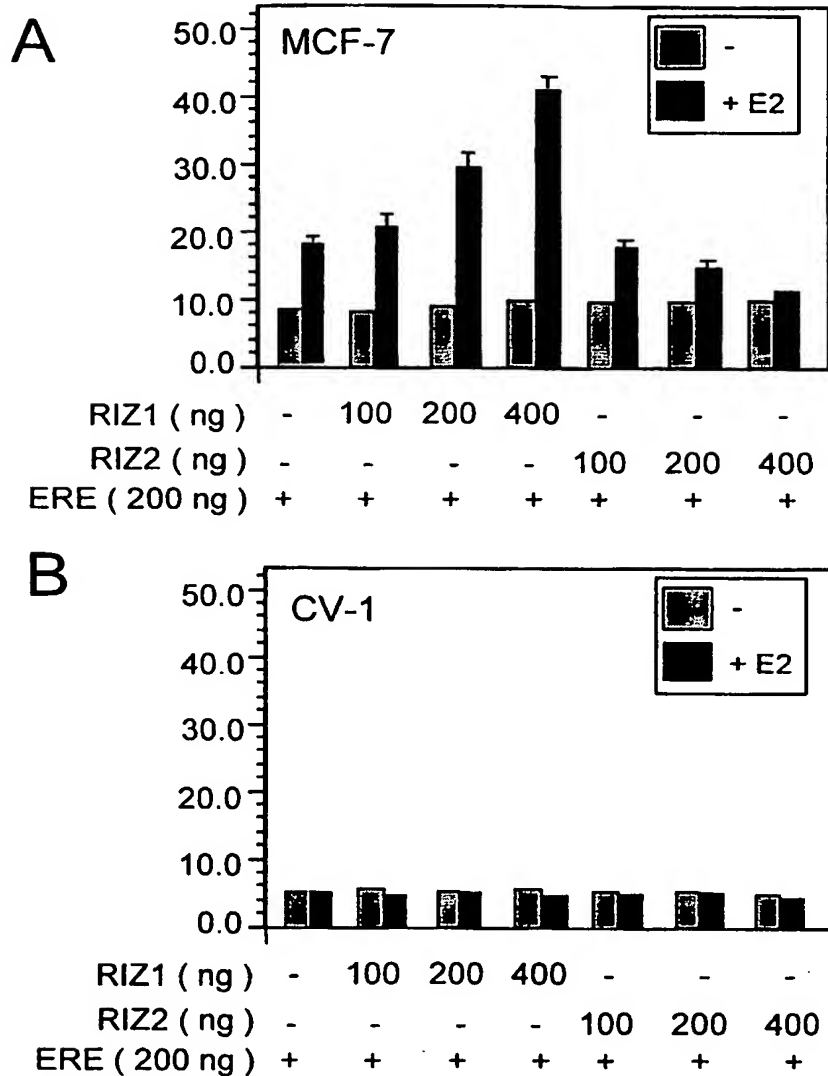
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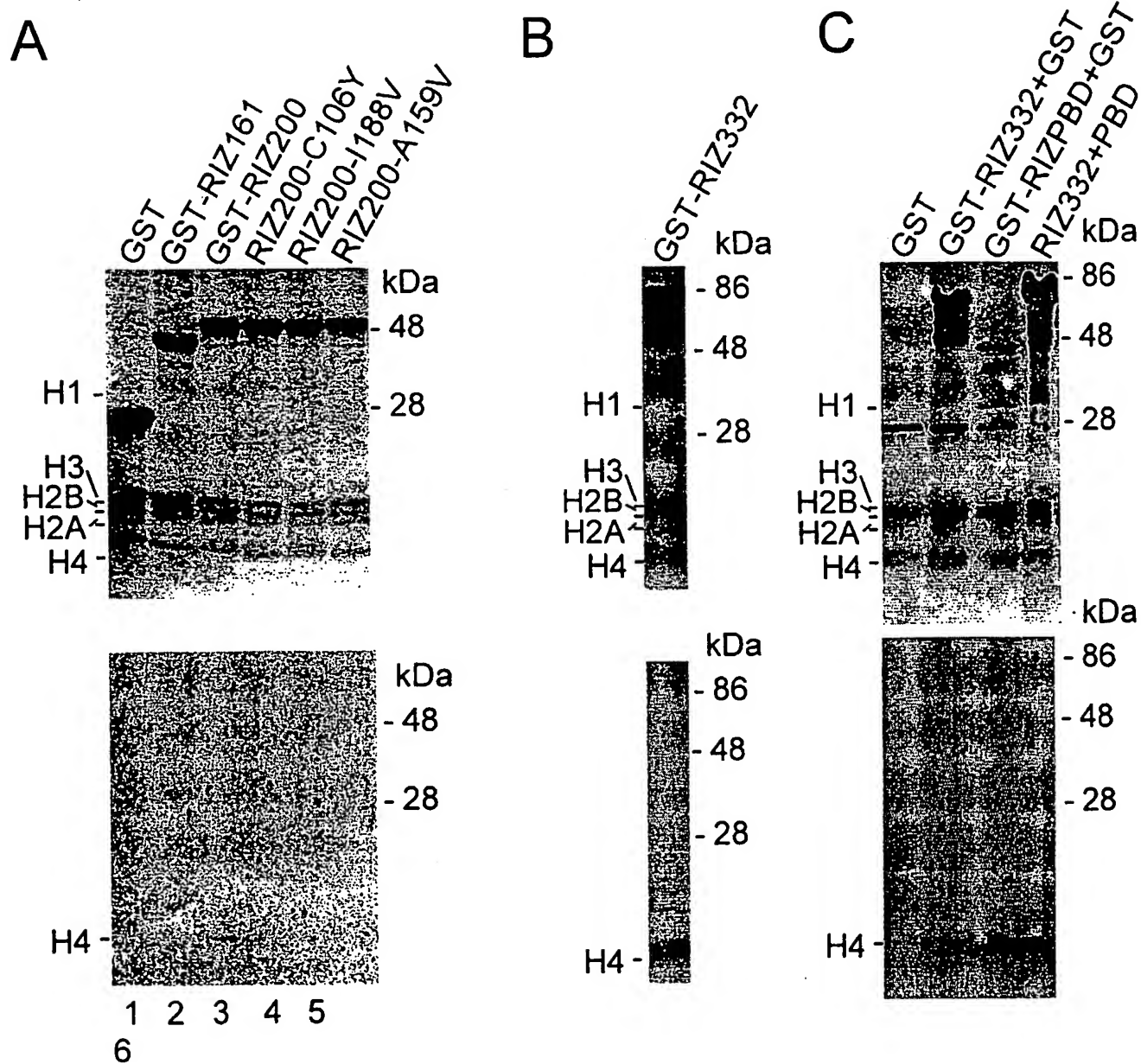
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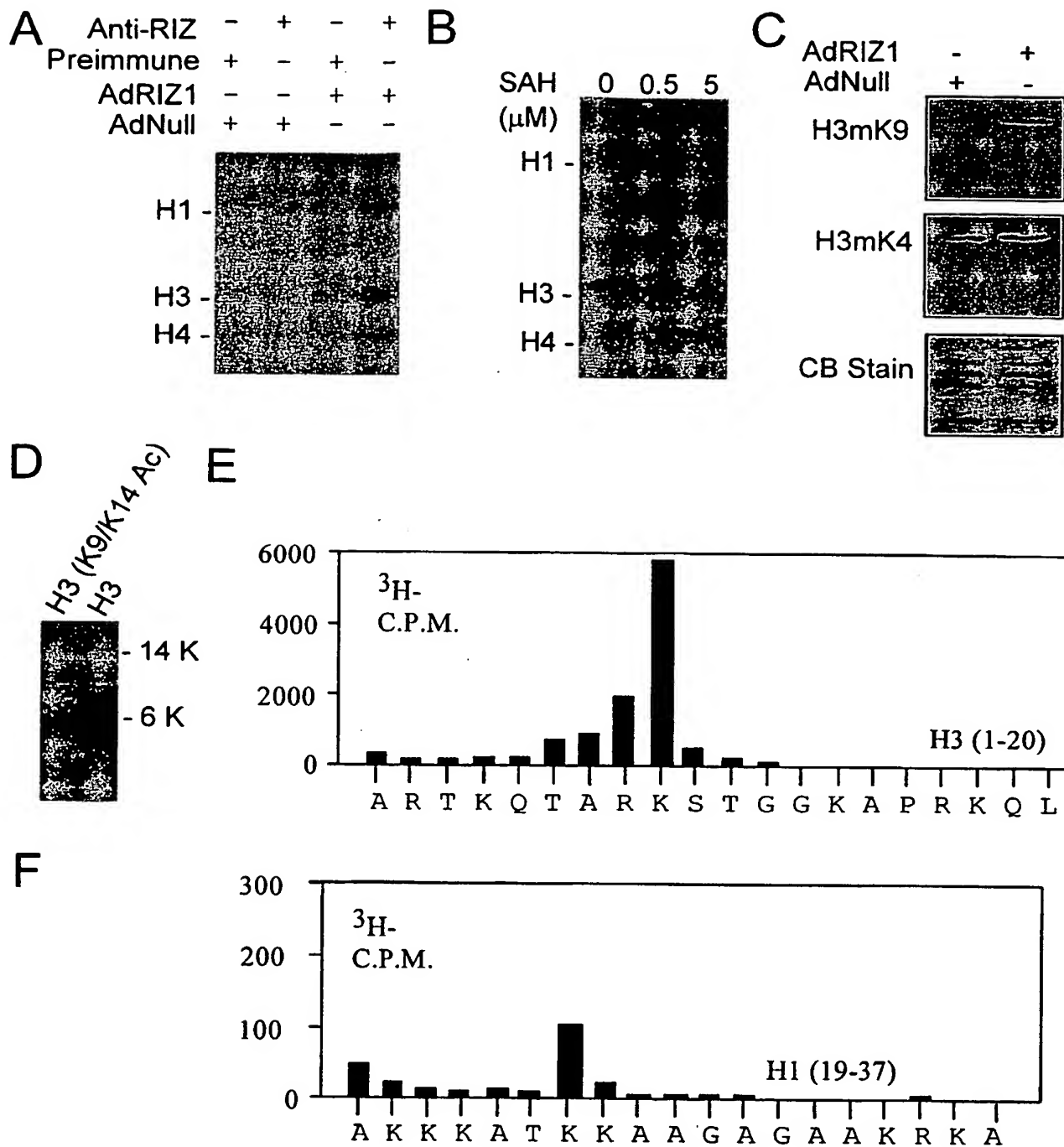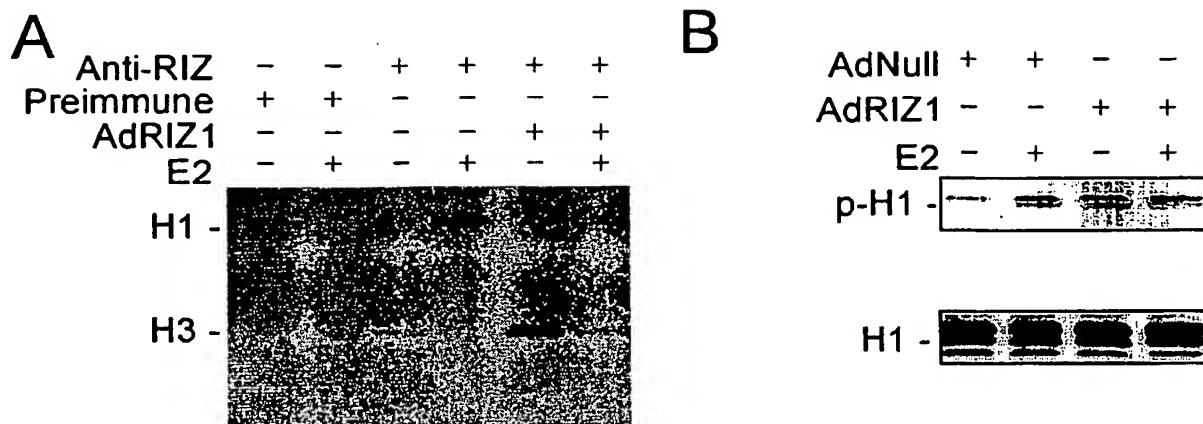
FIGURE 6

FIGURE 7

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Leu Pro Glu Asp Pro Leu Glu Thr Ser Lys Glu Glu Glu Glu Leu Asn
              1250                1255                1260
Asp Ser Ser Glu Glu Leu Tyr Thr Thr Ile Lys Ile Met Ala Ser Gly
1265                1270                1275                1280
Ile Lys Thr Lys Asp Pro Asp Val Arg Leu Gly Leu Asn Gln His Tyr
              1285                1290                1295
Pro Ser Phe Lys Pro Pro Pro Phe Gln Tyr His His Arg Asn Pro Met
              1300                1305                1310
Gly Ile Gly Val Thr Ala Thr Asn Phe Thr Thr His Asn Ile Pro Gln
              1315                1320                1325
Thr Phe Thr Thr Ala Ile Arg Cys Thr Lys Cys Gly Lys Gly Val Asp
              1330                1335                1340
Asn Met Pro Glu Leu His Lys His Ile Leu Ala Cys Ala Ser Ala Ser
1345                1350                1355                1360
Asp Lys Lys Arg Tyr Thr Pro Lys Lys Asn Pro Val Pro Leu Lys Gln
              1365                1370                1375
Thr Val Gln Pro Lys Asn Gly Val Val Val Leu Asp Asn Ser Gly Lys
              1380                1385                1390
Asn Ala Phe Arg Arg Met Gly Gln Pro Lys Arg Leu Asn Phe Ser Val
              1395                1400                1405
Glu Leu Ser Lys Met Ser Ser Asn Lys Leu Lys Leu Asn Ala Leu Lys
              1410                1415                1420
Lys Lys Asn Gln Leu Val Gln Lys Ala Ile Leu Gln Lys Asn Lys Ser
1425                1430                1435                1440
Ala Lys Gln Lys Ala Asp Leu Lys Asn Ala Cys Glu Ser Ser Ser His
              1445                1450                1455
Ile Cys Pro Tyr Cys Asn Arg Glu Phe Thr Tyr Ile Gly Ser Leu Asn
              1460                1465                1470
Lys His Ala Ala Phe Ser Cys Pro Lys Lys Pro Leu Ser Pro Pro Lys
              1475                1480                1485
Lys Lys Val Ser His Ser Ser Lys Lys Gly Gly His Ser Ser Pro Ala
              1490                1495                1500
Ser Ser Asp Lys Asn Ser Asn Ser Asn His Arg Arg Arg Thr Ala Asp
1505                1510                1515                1520
Ala Glu Ile Lys Met Gln Ser Met Gln Thr Pro Leu Gly Lys Thr Arg
              1525                1530                1535
Ala Arg Ser Ser Gly Pro Thr Gln Val Pro Leu Pro Ser Ser Ser Phe
              1540                1545                1550
Arg Ser Lys Gln Asn Val Lys Phe Ala Ala Ser Val Lys Ser Lys Lys
```

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                1555              1560              1565
Pro Ser Ser Ser Ser Leu Arg Asn Ser Ser Pro Ile Arg Met Ala Lys
    1570              1575              1580
Ile Thr His Val Glu Gly Lys Lys Pro Lys Ala Val Ala Lys Asn His
1585              1590              1595              1600
Ser Ala Gln Leu Ser Ser Lys Thr Ser Arg Ser Leu His Val Arg Val
                1605              1610              1615
Gln Lys Ser Lys Ala Val Leu Gln Ser Lys Ser Thr Leu Ala Ser Lys
            1620              1625              1630
Lys Arg Thr Asp Arg Phe Asn Ile Lys Ser Arg Glu Arg Ser Gly Gly
            1635              1640              1645
Pro Val Thr Arg Ser Leu Gln Leu Ala Ala Ala Ala Asp Leu Ser Glu
        1650              1655              1660
Asn Lys Arg Glu Asp Gly Ser Ala Lys Gln Glu Leu Lys Asp Phe Ser
1665              1670              1675              1680
Tyr Ser Leu Arg Leu Ala Ser Arg Cys Ser Pro Pro Ala Ala Pro Tyr
            1685              1690              1695
Ile Thr Arg Gln Tyr Arg Lys Val Lys Ala Pro Ala Ala Ala Gln Phe
        1700              1705              1710
Gln Gly Pro Phe Phe Lys Glu
        1715



<210> 2
<211> 1518
<212> PRT
<213> Homo sapiens

<400> 2
Met Arg Asp Ser Ala Glu Gly Pro Lys Glu Asp Glu Glu Lys Pro Ser
1               5                10               15
Ala Ser Ala Leu Glu Gln Pro Ala Thr Leu Gln Glu Val Ala Ser Gln
            20               25               30
Glu Val Pro Pro Glu Leu Ala Thr Pro Ala Pro Ala Trp Glu Pro Gln
            35               40               45
Pro Glu Pro Asp Glu Arg Leu Glu Ala Ala Ala Cys Glu Val Asn Asp
        50               55               60
Leu Gly Glu Glu Glu Glu Glu Glu Glu Glu Asp Glu Glu Glu Glu
65               70               75               80
Glu Asp Asp Asp Asp Asp Glu Leu Glu Asp Glu Gly Glu Glu Glu Ala
                85               90               95
Ser Met Pro Asn Glu Asn Ser Val Lys Glu Pro Glu Ile Arg Cys Asp
            100              105              110
Glu Lys Pro Glu Asp Leu Leu Glu Glu Pro Lys Thr Thr Ser Glu Glu
            115              120              125
Thr Leu Glu Asp Cys Ser Glu Val Thr Pro Ala Met Gln Ile Pro Arg
    130              135              140
Thr Lys Glu Glu Ala Asn Gly Asp Val Phe Glu Thr Phe Met Phe Pro
145              150              155              160
Cys Gln His Cys Glu Arg Lys Phe Thr Thr Lys Gln Gly Leu Glu Arg
            165              170              175
His Met His Ile His Ile Ser Thr Val Asn His Ala Phe Lys Cys Lys
            180              185              190
Tyr Cys Gly Lys Ala Phe Gly Thr Gln Ile Asn Arg Arg Arg His Glu
            195              200              205
```

```
Arg Arg His Glu Ala Gly Leu Lys Arg Lys Pro Ser Gln Thr Leu Gln
    210             215             220
Pro Ser Glu Asp Leu Ala Asp Gly Lys Ala Ser Gly Glu Asn Val Ala
225             230             235             240
Ser Lys Asp Asp Ser Ser Pro Pro Ser Leu Gly Pro Asp Cys Leu Ile
            245             250             255
Met Asn Ser Glu Lys Ala Ser Gln Asp Thr Ile Asn Ser Ser Val Val
        260             265             270
Glu Glu Asn Gly Glu Val Lys Glu Leu His Pro Cys Lys Tyr Cys Lys
        275             280             285
Lys Val Phe Gly Thr His Thr Asn Met Arg Arg His Gln Arg Arg Val
    290             295             300
His Glu Arg His Leu Ile Pro Lys Gly Val Arg Arg Lys Gly Gly Leu
305             310             315             320
Glu Glu Pro Gln Pro Pro Ala Glu Gln Ala Gln Ala Thr Gln Asn Val
            325             330             335
Tyr Val Pro Ser Thr Glu Pro Glu Glu Glu Gly Glu Ala Asp Asp Val
            340             345             350
Tyr Ile Met Asp Ile Ser Ser Asn Ile Ser Glu Asn Leu Asn Tyr Tyr
        355             360             365
Ile Asp Gly Lys Ile Gln Thr Asn Asn Asn Thr Ser Asn Cys Asp Val
    370             375             380
Ile Glu Met Glu Ser Ala Ser Ala Asp Leu Tyr Gly Ile Asn Cys Leu
385             390             395             400
Leu Thr Pro Val Thr Val Glu Ile Thr Gln Asn Ile Lys Thr Thr Gln
            405             410             415
Val Pro Val Thr Glu Asp Leu Pro Lys Glu Pro Leu Gly Ser Thr Asn
            420             425             430
Ser Glu Ala Lys Lys Arg Arg Thr Ala Ser Pro Pro Ala Leu Pro Lys
        435             440             445
Ile Lys Ala Glu Thr Asp Ser Asp Pro Met Val Pro Ser Cys Ser Leu
    450             455             460
Ser Leu Pro Leu Ser Ile Ser Thr Thr Glu Ala Val Ser Phe His Lys
465             470             475             480
Glu Lys Ser Val Tyr Leu Ser Ser Lys Leu Lys Gln Leu Leu Gln Thr
            485             490             495
Gln Asp Lys Leu Thr Pro Pro Ala Gly Ile Ser Ala Thr Glu Ile Ala
        500             505             510
Lys Leu Gly Pro Val Cys Val Ser Ala Pro Ala Ser Met Leu Pro Val
    515             520             525
Thr Ser Ser Arg Phe Lys Arg Arg Thr Ser Ser Pro Pro Ser Ser Pro
    530             535             540
Gln His Ser Pro Ala Leu Arg Asp Phe Gly Lys Pro Ser Asp Gly Lys
545             550             555             560
Ala Ala Trp Thr Asp Ala Gly Leu Thr Ser Lys Lys Ser Lys Leu Glu
            565             570             575
Ser His Ser Asp Ser Pro Ala Trp Ser Leu Ser Gly Arg Asp Glu Arg
        580             585             590
Glu Thr Val Ser Pro Pro Cys Phe Asp Glu Tyr Lys Met Ser Lys Glu
        595             600             605
Trp Thr Ala Ser Ser Ala Phe Ser Ser Val Cys Asn Gln Gln Pro Leu
    610             615             620
Asp Leu Ser Ser Gly Val Lys Gln Lys Ala Glu Gly Thr Gly Lys Thr
625             630             635             640
Pro Val Gln Trp Glu Ser Val Leu Asp Leu Ser Val His Lys Lys His
```

- 6 -

```
                          645                   650                   655
Cys Ser Asp Ser Glu Gly Lys Glu Phe Lys Glu Ser His Ser Val Gln
                660                   665                   670
Pro Thr Cys Ser Ala Val Lys Lys Arg Lys Pro Thr Thr Cys Met Leu
            675                   680                   685
Gln Lys Val Leu Leu Asn Glu Tyr Asn Gly Ile Asp Leu Pro Val Glu
        690                   695                   700
Asn Pro Ala Asp Gly Thr Arg Ser Pro Ser Pro Cys Lys Ser Leu Glu
705                   710                   715                   720
Ala Gln Pro Asp Pro Asp Leu Gly Pro Gly Ser Gly Phe Pro Ala Pro
                725                   730                   735
Thr Val Glu Ser Thr Pro Asp Val Cys Pro Ser Ser Pro Ala Leu Gln
                740                   745                   750
Thr Pro Ser Leu Ser Ser Gly Gln Leu Pro Pro Leu Leu Ile Pro Thr
            755                   760                   765
Asp Pro Ser Ser Pro Pro Pro Cys Pro Pro Val Leu Thr Val Ala Thr
        770                   775                   780
Pro Pro Pro Pro Leu Leu Pro Thr Val Pro Leu Pro Ala Pro Ser Ser
785                   790                       795                   800
Ser Ala Ser Pro His Pro Cys Pro Ser Pro Leu Ser Asn Ala Thr Ala
                805                   810                   815
Gln Ser Pro Leu Pro Ile Leu Ser Pro Thr Val Ser Pro Ser Pro Ser
            820                   825                   830
Pro Ile Pro Pro Val Glu Pro Leu Met Ser Ala Ala Ser Pro Gly Pro
            835                   840                   845
Pro Thr Leu Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Phe
        850                   855                   860
Ser Ser Ser Ser Ser Ser Ser Ser Pro Ser Pro Pro Pro Leu Ser Ala
865                   870                       875                   880
Ile Ser Ser Val Val Ser Ser Gly Asp Asn Leu Glu Ala Ser Leu Pro
                885                   890                   895
Met Ile Ser Phe Lys Gln Glu Glu Leu Glu Asn Glu Gly Leu Lys Pro
            900                   905                       910
Arg Glu Glu Pro Gln Ser Ala Ala Glu Gln Asp Val Val Val Gln Glu
            915                   920                   925
Thr Phe Asn Lys Asn Phe Val Cys Asn Val Cys Glu Ser Pro Phe Leu
            930                   935                   940
Ser Ile Lys Asp Leu Thr Lys His Leu Ser Ile His Ala Glu Glu Trp
945                   950                   955                   960
Pro Phe Lys Cys Glu Phe Cys Val Gln Leu Phe Lys Asp Lys Thr Asp
                965                   970                   975
Leu Ser Glu His Arg Phe Leu Leu His Gly Val Gly Asn Ile Phe Val
            980                   985                   990
Cys Ser Val Cys Lys Lys Glu Phe Ala Phe Leu Cys Asn Leu Gln Gln
        995                   1000                  1005
His Gln Arg Asp Leu His Pro Asp Lys Val Cys Thr His His Glu Phe
    1010                  1015                  1020
Glu Ser Gly Thr Leu Arg Pro Gln Asn Phe Thr Asp Pro Ser Lys Ala
1025                  1030                  1035                  1040
His Val Glu His Met Gln Ser Leu Pro Glu Asp Pro Leu Glu Thr Ser
                1045                  1050                  1055
Lys Glu Glu Glu Glu Leu Asn Asp Ser Ser Glu Glu Leu Tyr Thr Thr
                1060                  1065                  1070
Ile Lys Ile Met Ala Ser Gly Ile Lys Thr Lys Asp Pro Asp Val Arg
            1075                  1080                  1085
```

```
Leu Gly Leu Asn Gln His Tyr Pro Ser Phe Lys Pro Pro Phe Gln
    1090             1095             1100
Tyr His His Arg Asn Pro Met Gly Ile Gly Val Thr Ala Thr Asn Phe
1105             1110             1115             1120
Thr Thr His Asn Ile Pro Gln Thr Phe Thr Thr Ala Ile Arg Cys Thr
                1125             1130             1135
Lys Cys Gly Lys Gly Val Asp Asn Met Pro Glu Leu His Lys His Ile
            1140             1145             1150
Leu Ala Cys Ala Ser Ala Ser Asp Lys Lys Arg Tyr Thr Pro Lys Lys
        1155             1160             1165
Asn Pro Val Pro Leu Lys Gln Thr Val Gln Pro Lys Asn Gly Val Val
    1170             1175             1180
Val Leu Asp Asn Ser Gly Lys Asn Ala Phe Arg Arg Met Gly Gln Pro
1185             1190             1195             1200
Lys Arg Leu Asn Phe Ser Val Glu Leu Ser Lys Met Ser Ser Asn Lys
            1205             1210             1215
Leu Lys Leu Asn Ala Leu Lys Lys Lys Asn Gln Leu Val Gln Lys Ala
            1220             1225             1230
Ile Leu Gln Lys Asn Lys Ser Ala Lys Gln Lys Ala Asp Leu Lys Asn
        1235             1240             1245
Ala Cys Glu Ser Ser Ser His Ile Cys Pro Tyr Cys Asn Arg Glu Phe
    1250             1255             1260
Thr Tyr Ile Gly Ser Leu Asn Lys His Ala Ala Phe Ser Cys Pro Lys
1265             1270             1275             1280
Lys Pro Leu Ser Pro Pro Lys Lys Lys Val Ser His Ser Ser Lys Lys
            1285             1290             1295
Gly Gly His Ser Ser Pro Ala Ser Ser Asp Lys Asn Ser Asn Ser Asn
            1300             1305             1310
His Arg Arg Arg Thr Ala Asp Ala Glu Ile Lys Met Gln Ser Met Gln
        1315             1320             1325
Thr Pro Leu Gly Lys Thr Arg Ala Arg Ser Ser Gly Pro Thr Gln Val
    1330             1335             1340
Pro Leu Pro Ser Ser Ser Phe Arg Ser Lys Gln Asn Val Lys Phe Ala
1345             1350             1355             1360
Ala Ser Val Lys Ser Lys Lys Pro Ser Ser Ser Ser Leu Arg Asn Ser
            1365             1370             1375
Ser Pro Ile Arg Met Ala Lys Ile Thr His Val Glu Gly Lys Lys Pro
            1380             1385             1390
Lys Ala Val Ala Lys Asn His Ser Ala Gln Leu Ser Ser Lys Thr Ser
        1395             1400             1405
Arg Ser Leu His Val Arg Val Gln Lys Ser Lys Ala Val Leu Gln Ser
    1410             1415             1420
Lys Ser Thr Leu Ala Ser Lys Lys Arg Thr Asp Arg Phe Asn Ile Lys
1425             1430             1435             1440
Ser Arg Glu Arg Ser Gly Gly Pro Val Thr Arg Ser Leu Gln Leu Ala
            1445             1450             1455
Ala Ala Ala Asp Leu Ser Glu Asn Lys Arg Glu Asp Gly Ser Ala Lys
            1460             1465             1470
Gln Glu Leu Lys Asp Phe Ser Tyr Ser Leu Arg Leu Ala Ser Arg Cys
        1475             1480             1485
Ser Pro Pro Ala Ala Pro Tyr Ile Thr Arg Gln Tyr Arg Lys Val Lys
    1490             1495             1500
Ala Pro Ala Ala Ala Gln Phe Gln Gly Pro Phe Phe Lys Glu
1505             1510             1515
```

```
<210> 3
<211> 1706
<212> PRT
<213> Rattus sp.

<400> 3
Met His Gln Asn Thr Glu Ser Val Ala Ala Thr Glu Thr Leu Ala Glu
1               5                   10                  15
Val Pro Glu His Val Leu Arg Gly Leu Pro Glu Glu Val Arg Leu Phe
            20                  25                  30
Pro Ser Ala Val Asp Lys Thr Arg Ile Gly Val Trp Ala Thr Lys Pro
            35                  40                  45
Ile Leu Lys Gly Lys Lys Phe Gly Pro Phe Val Gly Asp Lys Lys Lys
        50                  55                  60
Arg Ser Gln Val Arg Asn Asn Val Tyr Met Trp Glu Val Tyr Tyr Pro
65                  70                  75                  80
Asn Leu Gly Trp Met Cys Ile Asp Ala Thr Asp Pro Glu Lys Gly Asn
                85                  90                  95
Trp Leu Arg Tyr Val Asn Trp Ala Cys Ser Gly Glu Glu Gln Asn Leu
            100                 105                 110
Phe Pro Leu Glu Ile Asn Arg Ala Ile Tyr Tyr Lys Thr Leu Lys Pro
            115                 120                 125
Ile Ala Pro Gly Glu Glu Leu Leu Val Trp Tyr Asn Gly Glu Asp Asn
        130                 135                 140
Pro Glu Ile Ala Ala Ala Ile Glu Glu Glu Arg Ala Ser Ala Arg Ser
145                 150                 155                 160
Lys Arg Ser Ser Pro Lys Ser Arg Arg Gly Lys Lys Lys Ser His Glu
                165                 170                 175
Asn Lys Asn Lys Gly Ile Arg Thr His Pro Thr Gln Leu Lys Ala Ser
            180                 185                 190
Glu Leu Asp Ser Thr Phe Ala Asn Met Arg Gly Ser Ala Glu Gly Pro
            195                 200                 205
Lys Glu Glu Asp Glu Arg Pro Leu Ala Ser Ala Pro Glu Gln Pro Ala
        210                 215                 220
Pro Leu Pro Glu Val Gly Asn Gln Asp Ala Val Pro Gln Val Ala Ile
225                 230                 235                 240
Pro Leu Pro Ala Cys Glu Pro Gln Pro Glu Val Asp Gly Lys Gln Glu
                245                 250                 255
Val Thr Asp Cys Glu Val Asn Asp Val Glu Glu Glu Glu Leu Glu Glu
            260                 265                 270
Glu Glu Glu Leu Glu Glu Glu Glu Glu Glu Glu Leu Gly Glu Asp Gly
            275                 280                 285
Val Glu Glu Ala Asp Met Pro Asn Glu Ser Ser Ala Lys Glu Pro Glu
        290                 295                 300
Ile Arg Cys Glu Glu Lys Pro Glu Asp Leu Leu Glu Glu Pro Gln Ser
305                 310                 315                 320
Met Ser Asn Glu Ala Arg Glu Asp Ser Pro Asp Val Thr Pro Pro Pro
                325                 330                 335
His Thr Pro Arg Ala Arg Glu Glu Ala Asn Gly Asp Val Leu Glu Thr
                340                 345                 350
Phe Met Phe Pro Cys Gln His Cys Glu Arg Lys Phe Ala Thr Lys Gln
            355                 360                 365
Gly Leu Glu Arg His Met His Ile His Ile Ser Thr Ile Asn His Ala
        370                 375                 380
```

```
Phe Lys Cys Lys Tyr Cys Gly Lys Arg Phe Gly Thr Gln Ile Asn Arg
385                     390                 395                 400
Arg Arg His Glu Arg Arg His Glu Thr Gly Leu Lys Arg Arg Pro Ser
                405                 410                 415
Met Thr Leu Gln Ser Ser Glu Asp Pro Asp Asp Gly Lys Gly Glu Asn
                420                 425                 430
Val Thr Ser Lys Asp Glu Ser Ser Pro Pro Gln Leu Gly Gln Asp Cys
                435             440                 445
Leu Ile Leu Asn Ser Glu Lys Thr Ser Gln Glu Val Leu Asn Ser Ser
        450                 455                 460
Phe Val Glu Glu Asn Gly Glu Val Lys Glu Leu His Pro Cys Lys Tyr
465                     470                 475                 480
Cys Lys Lys Val Phe Gly Thr His Thr Asn Met Arg Arg His Gln Arg
                485                 490                 495
Arg Val His Glu Arg His Leu Ile Pro Lys Gly Val Arg Arg Lys Gly
                500                 505                 510
Gly Leu Leu Glu Glu Pro Gln Pro Pro Ala Glu Gln Ala Pro Pro Ser
        515                 520                 525
Gln Asn Val Tyr Val Pro Ser Thr Glu Pro Glu Glu Glu Gly Glu Thr
        530                 535                 540
Asp Asp Val Tyr Ile Met Asp Ile Ser Ser Asn Ile Ser Glu Asn Leu
545                 550                 555                 560
Asn Tyr Tyr Ile Asp Gly Lys Ile Gln Thr Asn Ser Ser Thr Ser Asn
                565                 570                 575
Cys Asp Val Ile Glu Met Glu Ser Asn Ser Ala His Leu Tyr Gly Ile
                580                 585                 590
Asp Cys Leu Leu Thr Pro Val Thr Val Glu Ile Thr Gln Asn Ile Lys
        595                 600                 605
Ser Thr Gln Val Ser Val Thr Asp Asp Leu Leu Lys Asp Ser Pro Ser
    610                 615                 620
Ser Thr Asn Cys Glu Ser Lys Lys Arg Arg Thr Ala Ser Pro Pro Val
625                 630                 635                 640
Leu Pro Lys Ile Lys Thr Glu Thr Glu Ser Asp Ser Thr Ala Pro Ser
                645                 650                 655
Cys Ser Leu Ser Leu Pro Leu Ser Ile Ser Thr Ala Glu Val Val Ser
                660                 665                 670
Phe His Lys Glu Lys Gly Val Tyr Leu Ser Ser Lys Leu Lys Gln Leu
        675                 680                 685
Leu Gln Thr Gln Asp Lys Leu Thr Leu Pro Ala Gly Phe Ser Ala Ala
        690                 695                 700
Glu Ile Pro Lys Leu Gly Pro Val Cys Ala Ser Ala Pro Ala Ser Met
705                 710                 715                 720
Leu Pro Val Thr Ser Ser Arg Phe Lys Arg Arg Thr Ser Ser Pro Pro
                725                 730                 735
Ser Ser Pro Gln His Ser Pro Ala Leu Arg Asp Phe Gly Lys Pro Asn
        740                 745                 750
Asp Gly Lys Ala Ala Trp Thr Asp Thr Val Leu Thr Ser Lys Lys Pro
        755                 760                 765
Lys Leu Glu Ser Arg Ser Asp Ser Pro Ala Trp Ser Leu Ser Gly Arg
    770                 775                 780
Asp Glu Arg Glu Thr Gly Ser Pro Pro Cys Phe Asp Glu Tyr Lys Ile
785                 790                 795                 800
Ser Lys Glu Trp Ala Ala Ser Ser Thr Phe Ser Ser Val Cys Asn Gln
                805                 810                 815
Gln Pro Leu Asp Leu Ser Ser Gly Val Lys Gln Lys Ser Glu Gly Thr
```

- 10 -

```
                     820                      825                      830
     Gly Lys Thr Pro Val Pro Trp Glu Ser Val Leu Asp Leu Ser Val His
             835                      840                      845
     Lys Lys Pro Cys Asp Ser Glu Gly Lys Glu Phe Lys Glu Asn His Leu
         850                      855                      860
     Ala Gln Pro Ala Ala Lys Lys Lys Lys Pro Thr Thr Cys Met Leu Gln
     865                      870                      875                  880
     Lys Val Leu Leu Asn Glu Tyr Asn Gly Val Ser Leu Pro Thr Glu Thr
                     885                      890                      895
     Thr Pro Glu Val Thr Arg Ser Pro Ser Pro Cys Lys Ser Pro Asp Thr
                     900                      905                      910
     Gln Pro Asp Pro Glu Leu Gly Pro Asp Ser Ser Cys Ser Val Pro Thr
             915                      920                      925
     Ala Glu Ser Pro Pro Glu Val Val Gly Pro Ser Ser Pro Pro Leu Gln
         930                      935                      940
     Thr Ala Ser Leu Ser Ser Gly Gln Leu Pro Pro Leu Leu Thr Pro Thr
     945                      950                      955                  960
     Glu Pro Ser Ser Pro Pro Pro Cys Pro Pro Val Leu Thr Val Ala Thr
                     965                      970                      975
     Pro Pro Pro Pro Leu Leu Pro Thr Val Pro Leu Ser His Pro Ser Ser
                     980                      985                      990
     Asp Ala Ser Pro Gln Gln Cys Pro Ser Pro Phe Ser Asn Thr Thr Ala
             995                      1000                     1005
     Gln Ser Pro Leu Pro Ile Leu Ser Pro Thr Val Ser Pro Ser Pro Ser
         1010                     1015                     1020
     Pro Ile Pro Pro Val Glu Pro Leu Met Ser Ala Ala Ser Pro Gly Pro
     1025                     1030                     1035                 1040
     Pro Thr Leu Ser Ser Ser Ser Ser Ser Ser Ser Ser Phe Pro Ser Ser
                     1045                     1050                     1055
     Ser Cys Ser Ser Thr Ser Pro Ser Pro Pro Pro Leu Ser Ala Val Ser
                     1060                     1065                     1070
     Ser Val Val Ser Ser Gly Asp Asn Leu Glu Ala Ser Leu Pro Ala Val
             1075                     1080                     1085
     Thr Phe Lys Gln Glu Glu Ser Glu Ser Glu Gly Leu Lys Pro Lys Glu
             1090                     1095                     1100
     Glu Ala Pro Pro Ala Gly Gly Gln Ser Val Val Gln Glu Thr Phe Ser
     1105                     1110                     1115                 1120
     Lys Asn Phe Ile Cys Asn Val Cys Glu Ser Pro Phe Leu Ser Ile Lys
                     1125                     1130                     1135
     Asp Leu Thr Lys His Leu Ser Val His Ala Glu Glu Trp Pro Phe Lys
                     1140                     1145                     1150
     Cys Glu Phe Cys Val Gln Leu Phe Lys Val Lys Thr Asp Leu Ser Glu
             1155                     1160                     1165
     His Arg Phe Leu Leu His Gly Val Gly Asn Ile Phe Val Cys Ser Val
         1170                     1175                     1180
     Cys Lys Lys Glu Phe Ala Phe Leu Cys Asn Leu Gln Gln His Gln Arg
     1185                     1190                     1195                 1200
     Asp Leu His Pro Asp Glu Val Cys Thr His His Glu Phe Glu Ser Gly
                     1205                     1210                     1215
     Thr Leu Arg Pro Gln Asn Phe Thr Asp Pro Ser Lys Ala Asn Val Glu
             1220                     1225                     1230
     His Met Pro Ser Leu Pro Glu Glu Pro Leu Glu Thr Ser Arg Glu Glu
             1235                     1240                     1245
     Glu Leu Asn Asp Ser Ser Glu Glu Leu Tyr Thr Thr Ile Lys Ile Met
         1250                     1255                     1260
```

Ala Ser Gly Ile Lys Thr Lys Asp Pro Asp Val Arg Leu Gly Leu Asn
1265   1270   1275   1280
Gln His Tyr Pro Ser Phe Lys Pro Pro Phe Gln Tyr His His Arg
              1285   1290   1295
Asn Pro Met Gly Ile Gly Val Thr Ala Thr Asn Phe Thr Thr His Asn
              1300   1305   1310
Ile Pro Gln Thr Phe Thr Thr Ala Ile Arg Cys Thr Lys Cys Gly Lys
              1315   1320   1325
Gly Val Asp Asn Met Pro Glu Leu His Lys His Ile Leu Ala Cys Ala
              1330   1335   1340
Ser Ala Ser Asp Lys Lys Arg Tyr Thr Pro Lys Lys Asn Pro Val Pro
1345   1350   1355   1360
Leu Lys Gln Thr Val Gln Pro Lys Asn Gly Val Val Val Leu Asp Asn
              1365   1370   1375
Ser Gly Lys Asn Ala Phe Arg Arg Met Gly Gln Pro Lys Arg Leu Ser
              1380   1385   1390
Phe Asn Val Glu Leu Gly Lys Met Ser Pro Asn Lys Leu Lys Leu Ser
              1395   1400   1405
Ala Leu Lys Lys Lys Asn Gln Leu Val Gln Lys Ala Ile Leu Gln Lys
              1410   1415   1420
Asn Arg Ala Ala Lys Gln Lys Ala Asp Leu Arg Asp Thr Ser Glu Ala
1425   1430   1435   1440
Ser Ser His Ile Cys Pro Tyr Cys Asp Arg Glu Phe Thr Tyr Ile Gly
              1445   1450   1455
Ser Leu Asn Lys His Ala Ala Phe Ser Cys Pro Lys Lys Pro Leu Ser
              1460   1465   1470
Pro Ser Lys Arg Lys Val Ser His Ser Ser Lys Lys Gly Gly His Ala
              1475   1480   1485
Ser Ser Ser Ser Ser Asp Arg Asn Ser Ser Cys His Pro Arg Arg Arg
              1490   1495   1500
Thr Ala Asp Thr Glu Ile Lys Met Gln Ser Thr Gln Ala Pro Leu Gly
1505   1510   1515   1520
Lys Thr Arg Ala Arg Ser Thr Gly Pro Ala Gln Ala Ser Leu Pro Ser
              1525   1530   1535
Ser Ser Phe Arg Ser Arg Gln Asn Val Lys Phe Ala Ala Ser Val Lys
              1540   1545   1550
Ser Lys Lys Ala Ser Ser Ser Ser Leu Arg Asn Ser Ser Pro Ile Arg
              1555   1560   1565
Met Ala Lys Ile Thr His Val Glu Gly Lys Lys Pro Lys Ala Val Ala
              1570   1575   1580
Lys Ser His Ser Ala Gln Leu Ser Ser Lys Ser Ser Arg Gly Leu His
1585   1590   1595   1600
Val Arg Val Gln Lys Ser Lys Ala Val Ile Gln Ser Lys Thr Ala Leu
              1605   1610   1615
Ala Ser Lys Arg Arg Thr Asp Arg Phe Ile Val Lys Ser Arg Glu Arg
              1620   1625   1630
Ser Gly Gly Pro Ile Thr Arg Ser Leu Gln Leu Ala Ala Ala Ala Asp
              1635   1640   1645
Leu Ser Glu Ser Arg Arg Glu Asp Ser Ser Ala Arg His Glu Leu Lys
              1650   1655   1660
Asp Phe Ser Tyr Ser Leu Arg Leu Ala Ser Arg Cys Gly Ser Ser Thr
1665   1670   1675   1680
Ala Ser Tyr Ile Thr Arg Gln Cys Arg Lys Val Lys Ala Ala Ala Ala
              1685   1690   1695
Thr Pro Phe Gln Gly Pro Phe Leu Lys Glu

<210> 4
<211> 1506
<212> PRT
<213> Rattus sp.

<400> 4

Met	Arg	Gly	Ser	Ala	Glu	Gly	Pro	Lys	Glu	Glu	Asp	Glu	Arg	Pro	Leu
1				5					10					15	

Met Arg Gly Ser Ala Glu Gly Pro Lys Glu Glu Asp Glu Arg Pro Leu
 1   5   10   15
Ala Ser Ala Pro Glu Gln Pro Ala Pro Leu Pro Glu Val Gly Asn Gln
            20   25   30
Asp Ala Val Pro Gln Val Ala Ile Pro Leu Pro Ala Cys Glu Pro Gln
            35   40   45
Pro Glu Val Asp Gly Lys Gln Glu Val Thr Asp Cys Glu Val Asn Asp
    50   55   60
Val Glu Glu Glu Glu Leu Glu Glu Glu Glu Leu Glu Glu Glu Glu
65   70   75   80
Glu Glu Glu Leu Gly Glu Asp Gly Val Glu Glu Ala Asp Met Pro Asn
                85   90   95
Glu Ser Ser Ala Lys Glu Pro Glu Ile Arg Cys Glu Glu Lys Pro Glu
            100   105   110
Asp Leu Leu Glu Glu Pro Gln Ser Met Ser Asn Glu Ala Arg Glu Asp
        115   120   125
Ser Pro Asp Val Thr Pro Pro Pro His Thr Pro Arg Ala Arg Glu Glu
    130   135   140
Ala Asn Gly Asp Val Leu Glu Thr Phe Met Phe Pro Cys Gln His Cys
145   150   155   160
Glu Arg Lys Phe Ala Thr Lys Gln Gly Leu Glu Arg His Met His Ile
            165   170   175
His Ile Ser Thr Ile Asn His Ala Phe Lys Cys Lys Tyr Cys Gly Lys
            180   185   190
Arg Phe Gly Thr Gln Ile Asn Arg Arg Arg His Glu Arg Arg His Glu
            195   200   205
Thr Gly Leu Lys Arg Arg Pro Ser Met Thr Leu Gln Ser Ser Glu Asp
    210   215   220
Pro Asp Asp Gly Lys Gly Glu Asn Val Thr Ser Lys Asp Glu Ser Ser
225   230   235   240
Pro Pro Gln Leu Gly Gln Asp Cys Leu Ile Leu Asn Ser Glu Lys Thr
            245   250   255
Ser Gln Glu Val Leu Asn Ser Ser Phe Val Glu Glu Asn Gly Glu Val
            260   265   270
Lys Glu Leu His Pro Cys Lys Tyr Cys Lys Lys Val Phe Gly Thr His
            275   280   285
Thr Asn Met Arg Arg His Gln Arg Arg Val His Glu Arg His Leu Ile
    290   295   300
Pro Lys Gly Val Arg Arg Lys Gly Gly Leu Leu Glu Glu Pro Gln Pro
305   310   315   320
Pro Ala Glu Gln Ala Pro Pro Ser Gln Asn Val Tyr Val Pro Ser Thr
            325   330   335
Glu Pro Glu Glu Glu Gly Glu Thr Asp Asp Val Tyr Ile Met Asp Ile
            340   345   350
Ser Ser Asn Ile Ser Glu Asn Leu Asn Tyr Tyr Ile Asp Gly Lys Ile
            355   360   365

                          - 13 -

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Asn Ser Ala His Leu Tyr Gly Ile Asp Cys Leu Leu Thr Pro Val Thr
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Asp Leu Leu Lys Asp Ser Pro Ser Ser Thr Asn Cys Glu Ser Lys Lys
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Glu Ser Asp Ser Thr Ala Pro Ser Cys Ser Leu Ser Leu Pro Leu Ser
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Cys Ala Ser Ala Pro Ala Ser Met Leu Pro Val Thr Ser Ser Arg Phe
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Pro Cys Phe Asp Glu Tyr Lys Ile Ser Lys Glu Trp Ala Ala Ser Ser
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Thr Phe Ser Ser Val Cys Asn Gln Gln Pro Leu Asp Leu Ser Ser Gly
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Val Lys Gln Lys Ser Glu Gly Thr Gly Lys Thr Pro Val Pro Trp Glu
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Ser Val Leu Asp Leu Ser Val His Lys Lys Pro Cys Asp Ser Glu Gly
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Lys Pro Thr Thr Cys Met Leu Gln Lys Val Leu Leu Asn Glu Tyr Asn
        675   680   685
Gly Val Ser Leu Pro Thr Glu Thr Thr Pro Glu Val Thr Arg Ser Pro
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Ser Pro Cys Lys Ser Pro Asp Thr Gln Pro Asp Pro Glu Leu Gly Pro
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Phe Ile Val Lys Ser Arg Glu Arg Ser Gly Gly Pro Ile Thr Arg Ser
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Organization
International Bureau

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PCT

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(54) Title: SCREENING, DIAGNOSTIC AND THERAPEUTIC METHODS RELATING TO RIZ

(57) Abstract: The invention provides a method of screening for a compound that modulates RIZ histone methyltransferase (HMT) activity, by contacting a RIZ or RIZ fragment having HMT activity with one or more candidate compounds, and determining histone methyltransferase activity of the contacted RIZ or RIZ fragment. Also provided is a method of screening for a compound that modulates progesterone receptor (PR) activity, by providing a RIZ1 modulatory compound, and determining the ability of the RIZ1 modulatory compound to modulate PR activity. Further provided is a method of identifying an individual with an estrogen receptor positive (ER+) tumor having a reduced likelihood of responding to endocrine therapy. The method involves determining the RIZ1 status of the tumor, wherein an abnormal RIZ1 status identifies the individual as an individual with a reduced likelihood of responding to endocrine therapy.

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/68; G01N 33/48

US CL : 435/4, 6; 436/63, 64

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 6; 436/63, 64

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BUYSE et al. The Retinoblastoma Protein Binds to RIZ, a Zinc-Finger Protein that Shares an Epitope with the Adenovirus E1A Protein. Proc. Natl. Acad. Sci. USA. May 1995, Vol. 92, pates 4467-4471, see abstract.	1-24
Y	STEELE-PERKINS et al. Tumor Formation and Inactivation of RIZ1, an Rb-Binding Member of a Nuclear Protein-Methyltransferase Superfamily. Genes & Development. 2001, Vol. 15, pages 2250-2262, especially 2250-2251.	1-9
Y,E	US 6,555,329 B2 (JENUWEIN et al) 29 April 2003 (29.04.2003), column 53 - column 56.	1-9

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent published on or after the international filing date
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"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 June 2004 (27.06.2004)	23 JUL 2004

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450	Anne Holleran f. Roberts for
Facsimile No. (703) 872-9306	Telephone No. (571) 272-1600

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INTERNATIONAL SEARCH REPORT

Continuation of B. FIELDS SEARCHED Item 3:
Medline, US Patents database in EAST
search terms: RIZ, methyltransferase, histone, progesterone, estrogen

Form PCT/ISA/210 (second sheet) (July 1998)